表❶ ヒトES細胞用培地（文献1，17より）

最終濃度	ストック	Invitrogen番号
80% Knockout DMEM *		10829-018
20% Knockout SR		10828-028
1% non essential amino acid solution	100x MEM non essential amino acid solution	11140-035
1mM L-glutamine	0.146g in 10mL PBS	21-51-016
0.1mM β mercaptoethanol	14.3M β mercaptoethanol	Sigma M-7154
4ng/mL human bFGF **	2 μg/mL in PBS with 0.1% BSA	13256-029

＊：DM/F12が使用されることも多い。
＊＊：施設によって使用濃度は少しずつ異なっているので，実際に培養を開始する際には，その組織に添付されているプロトコルを参照のこと。

【手順】
① L-glutamine/β-mercaptoethanol液の作製
10mLのCMF-PBSに，0.146gのL-glutamineを15mLチューブに入れる
7μLのβ-mercaptoethanolを入れてよく混ぜる
② 225mLのフィルターユニットに以下を入れる

Knockout DMEM	160mL
Knockout SR	40mL
L-glutamine/β-mercaptoethanol液	2mL
100x non-essential amino acid solution	2mL
human bFGF	400μL

③ フィルターする
④ 4℃にて保存し，2週間以内に使用する

使用する血清のロットによって分化誘導されてくる組織が異なることはしばしばみられる。さらに，プリオンやウイルスなどの感染体を含んでいる可能性がある。KSRは無血清であると言われているが，ロット差のある動物由来成分を含む。品質の安定性や病原体をできるだけ排除するためにも，未知の成分を含まない無血清培養が望ましいと考えられる。

Ⅱ．defined mediumの開発

1．サプリメント

無血清培養とは，既知の成分よりなる合成培地を用いた培養条件，すなわちchemically defined serum-free culture[5]であり，単に血清を除いた基礎培地のみによる培養ではない。1975年にSatoら[6]が，血清の役割とは，それに含まれるホルモン，増殖因子，接着因子などが細胞の増殖を促進することであり，これらの因子を基礎培地に加えることにより血清を代替できることを提言した。1979年にBottensteinら[7]により，神経細胞の無血清培養用として，N2サプリメント（インスリン，トランスフェリン，プロゲステロン，セレニウム，プトレッシン）が開発された。その後，N2サプリメントは5因子（インスリン，トランスフェリン，エタノールアミン，2-メルカプトエタノール，セレン酸）あるいは6因子（+オレイン酸）に改良された。その結果，神経細胞だけでなく様々な細胞の無血清培養を行うことが可能となり，血清添加の条件で培養されていた細胞が既知の因子により培養できるようになった[8-10]。このような既知の組成からなる培養液を用いることにより，細胞の増殖や分化に必要な因子の要求性など細胞の機能が正確に解析でき，機能性培養系とも呼ばれている。一方，1993年にPriceらのグループによって，神経細胞の無血清培養用にインスリンを含む20因子から構成されているB27サプリメント[11]が開発されたが，濃度は公開されていない。さらに1998年にPriceらは，KSRを開発した。上述したように，現在ヒトES細胞用培地のサプリメントとして広く用いられているが，組成は公開されていない。

2．ヒトES細胞用無血清培地　TeSR1培地とhESF9

これまでに，ヒトES細胞用の培養条件は，市販品も含めて10例前後報告されている。しかし，完全に組成が既知の因子からなり公開されているのは，Ludwigらによる TeSR1 培地[12]と，筆者らが開発したhESF9[13]のみである。TeSR1培地（StemCell Technologies USAより販売）は，DM/F12を基礎培地として改良し，pipecolic acid，インスリン，トランスフェリン，メルカプトエタノール，脂質群，GABA，FGF-2，TGF-β，

Wnt シグナルを活性化する LiCl₂ などを加えた条件となっている。接着因子はマトリゲルの代わりに、フィブロネクチン、ラミニン、ビトロネクチン、IV型コラーゲンを混合したものを用いている。多くの細胞株で培養が安定して培養が可能であるようである。

一方、筆者はマウス ES 細胞の未分化性を維持することのできる無血清培養条件 ESF7 を開発した[6,7]（細胞科学研究所より販売）。さらに、英国シェフィールド大学幹細胞センター Andrews 教授らとの共同研究により、ヒト ES 細胞用の無血清培地 hESF9 を開発した[8]（特許申請中）。hESF9 は、筆者らが独自に開発したヒト ES 用 ESF（細胞科学研究所より販売）を基礎培地として、上述の 6 ファクターに、FGF-2 とヘパリンのみを加え、接着因子としては I 型コラーゲンを用いている。非常にシンプルな組成となっているため、添加因子の影響が高感度に解析できる。

安定した培養維持が行えるのは TeSR1 培地と思われるが、増殖因子や分化誘導因子などの影響や細胞内シグナルなどの解析には hESF9 が向いているだろう。それぞれの目的に合わせて使用するのが望ましいと考える。

Ⅲ．標準化培養

現在、英国やアメリカ合衆国などを中心として、臨床で使用するためのヒト ES 細胞株の樹立、品質保証などにわたる取扱基準規格の構築が行われている。臨床用だけでなく非臨床用に使用されるとしても、臨床用に準じた方法で分離・維持されるべきであろう。臨床用ヒト ES 細胞用の培養液は、すべての組成を明らかにして、異常などができるだけ発生しないよう安全性や危険性を多くの研究者により多角的に検討されるべきであると考える。しかし、培養の基礎知識のないまま使用されるために、培養液の機能を十分に発揮できていないことも多い。例えば、ボトルにすべての添加因子が添加されているような場合、そのボトルを繰り返し温めて使用する研究者が多い。しかし、添加されている増殖因子などのタンパク質は温められることに劣化し、CO_2 が抜けて pH は上昇する。幹細胞は、弱酸性には強いがアルカリ性には弱い[9]。このような培養液を用いていれば、幹細胞の品質は維持できない。様々な問題を克服して高品質の細胞を安定して供給するためには、培養細胞をただの材料として扱うのではなく、体系的な細胞培養学の中で標準的培養法を確立していかなくては難しいだろう。

❖ おわりに

ヒト ES 細胞は、その樹立の方法や培養液が研究室により異なることもあり、株間の差が大きく、ヒト ES 細胞に関連する報告が追試できないことも多く、国際的に標準化が求められている。シェフィールド大学 Andrews 教授がリーダーとして推進している International Human ES Cell Initiatives（ISCI）プロジェクトでは、日本で樹立された細胞も含めて世界中で研究用に使用されている 59 種類のヒト ES 細胞株の特徴を比較し、2007 年 6 月に Nature Biotechnology に報告をした[10]。現時点では、海外においてもヒト ES 細胞の実用化は実施されていない。しかし、分化細胞を含めヒト ES 細胞製剤が患者に導入される日は遠くないだろう。国内においても、ヒト ES 細胞、iPS 細胞の培養法と応用法、その安全性と危険性を多くの研究者によって検討されることが望まれる。

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日本におけるヒトES、iPS細胞研究標準化：その2　分化能の評価

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要旨　1998年にヒト胚性幹（ES）細胞が樹立され、2007年にはヒト人工多能性幹（iPS）細胞が開発された。これらの細胞を用いて、発生過程におけるメカニズムの解明、あるいは再生医療や創薬などへの応用にむけて、国内外で研究が盛んに進められている。ヒトES細胞は受精卵の内部細胞塊由来であることから、成体組織を構成するすべての細胞に分化する可能性をもつ。また、ヒトiPS細胞はヒトES細胞様に分化能を有している。これらのことから、ヒトES/iPS細胞を樹立する際には、多能性の確認が重要であると言われている。この総説では、ヒトES/iPS細胞の分化能の評価法について概説する。

キーワード：　ヒトES細胞、ヒトiPS細胞、分化能、胚様体

序　　文

1998年Thomsonらにより[1]ヒトES細胞が樹立され、日本においては2004年に中辻らのグループにより[2,3]ヒトES細胞株が樹立された。2006年山中らのグループによりマウス人工多能性幹細胞（iPS）細胞[4]、2007年ヒトiPS細胞の樹立が発表された[5]。2009年8月には文部科学省によるヒトES細胞使用研究指針が緩和され、国内でもES/iPS細胞研究がさかんとなってきた。

前回の総説でも述べたが[7]、ヒトES/iPS細胞は従来の一般的な培養細胞とは異なる点が多い。また、研究室間による研究結果や技術の差も大きい。細胞株間の差も大きい。英国シェフィールド大学Andrews教授がリーダーとして推進しているInternational human ES cell initiatives（ISCI）プロジェクトでは、日本を含めた世界11カ国の研究者らが共同でヒトES細胞株の特徴を比較し、ヒトES細胞研究の標準化が進められている[8,9]。これまでにISCIプロジェクトでは、細胞の登録、樹立の方法、未分化／分化マーカーの発現などの解析方法と結果をwebpageに公表している。（http://www.stemcellforum.org/isci_project/download_data.cfm）。ヒトiPS細胞は、ヒトES細胞と同様の特徴を持つことから、これらの解析方法は、iPS細胞にも応用されるべきであるとの認識が持たれている。ヒトES細胞の培養経験がない場合には、上記のサイトを参考にしてヒトES細胞の一般的な基準を把握する必要があるだろう。ES/iPS細胞の未分化性は不安定であり、また培養している間に形質が変化する。使用しているES/iPS細胞が標準通り培養で

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きているかどうか、随時確認を行う必要がある。異常クローンが出現した場合、3継代でほとんどの細胞集団が入れ替わると言われており、5代に1度の確認が望ましいと言われている。細胞資源研究室では、継代10代ごとに確認を行っている。

ヒトES細胞研究は、海外では経験値として語り伝えられる事項が多くあり、それらは論文となってデータに示されることは少ない。ヒトES細胞研究経験の少ない日本においては、ほとんどこれらの経験が伝えられていないのが現状である。そこで、正確なデータとして表示できないような内容も含めて、今回はヒトES/iPS細胞の分化能について概説する。

(1) 分化能について

ヒトES細胞は、受精卵の内部細胞塊由来であることから、成体組織を構成するすべての細胞に分化する可能性をもつため、ヒトES/iPS細胞を樹立する際には、多能性の確認が重要であると言われる。確かに、ヒトES/iPS細胞の未分化性維持機構解明、ヒトの発生過程の解明などを行うためには多能性であることは必要不可欠な条件であろう。一方、再生医療や創薬などへの応用を目指す場合、目的の分化細胞が得られればよく、必ずしも多能性を持つ必要はないのではないだろうか。ISCIプロジェクトに参加する海外のトップ研究者達は、特定の細胞に分化することが可能であればよい、と口にしている。情報として必要なのは、株の分化傾向や目的細胞への分化効率だろう。他の細胞株と比較して、効率よく神経に分化する細胞株はどれか？　心筋に分化しやすい細胞株どれか？産業応用にはこれらのことがもっとも求められている情報だろう。このような分化傾向の評価は、一般的にES/iPS細胞を凝集させて胚様体（Embryoid body; EB）を作成させて分化誘導し、1週間から3ヶ月程度培養を行って分化マーカー遺伝子の発現を解析している[9,10]。ただし、EB形成に用いる細胞の状態が分化に影響を与える。また、培養に使用する血清によっても分化誘導効率が異なるといわれている。再生医療や創薬応用のためには、誤差をできるだけ少なくして分化能を評価することが必要である。これまでに様々な分化細胞へのプロトコールが報告されているが、必ずしも再現性の高いわけではない。今後、それぞれの分化誘導法が検討され、各種プロトコールを用いた分化能評価が行われることになるだろう。

(2) EB作成時の培養条件

一般的には、通常の継代通り細胞を分散し、細胞接着のための処理のされていないペトリディッシュに播種する。その際に使用する培地は、（i）FGF-2を含むES/iPS細胞未分化維持用培地、（ii）FGF-2を含まないES/iPS細胞未分化維持用培地、（iii）10%ウシ血清・低グルコースDMEM＋2－メルカプトエタノールなどの条件が用いられる。（iii）の血清を含む培地を用いた場合、細胞死が多く見られ、EBを形成しないことも多い。一般的には、（ii）のFGF-2を含まない未分化維持培地を用いるとされているが、（i）のFGF-2を含む未分化維持培地を用いると、安定してEBが作成できることが多いと言われる。筆者の経験では、これら3種の培養条件のうち、（i）の条件が効率よくEBを形成する株が多い。EBを形成させた後は、徐々に（iii）の血清を含む培地に変えていき、1週間EBを培養した後は、2－メルカプトエタノールを含まない培地で培養を行っている（表1）。

(3) EBの大きさ

EBの大きさを調整することは難しい（図1）。スフェロイド作成用の96ウェルプレート（スミロンスフェロイド）を使用すると、ある程度の大きさはそろえられるが、ウェル内で小さなEBを複数形成することなどもある。また、培養皿底面に小さなマイクロウェルが刻まれているようなプ

表1．JCRB におけるヒト ES、iPS 細胞の EB 形成の手順*

手　　順	時　間
培地を吸引。	
1 unit/ml Dispase⑥（Roche／解凍後 3 日以内に使用）を 1 ml 入れる。	
37°C・CO_2 インキュベーターに入れてインキュベーション。	3~10 min⑥
Dispase を吸引。	
hES 培地 10 ml を入れて、10 ml ピペットをつけたピペットエイド（強にする）で培地を吹きかけるようにしてコロニーをはがす（できるだけ回数を少なくする。2 回程度のピペッティングでコロニーがはがれないような場合は、セルスクレーパーを使用してコロニーをはがす）。	
顕微鏡でコロニーの分散状態を確認する。	
15 ml チューブに細胞浮遊液を入れて、300 rpm にて遠心（大きいコロニーのみを回収する）。	2 min
新しい hES 培地を入れて細胞浮遊液とする（ピペッティングはしない）。	
10 cm 細菌培養用ペトリディッシュに 3–5 × 10^6 にて播種	
顕微鏡でコロニーの分散状態を確認。	
CO_2 インキュベーターに入れて培養。	3 日間
EB用培地を必要量準備	
37°C ウォーターバスで培地を温める。	5 分
EB と培地を合わせてチューブに回収し、EB 用培地**を加える。	
浮遊細胞培養用 25 cm^2 フラスコ 5 つに分種	
CO_2 インキュベーターに入れて、培養。	
1 週間に 2 回、2-ME を含む培地で交換をおこない、その後は 2-ME なしの培地で交換をおこなう	
DNA/RNA 回収***	

* （林田、小澤、松村、その他、論文投稿準備中）
** 10%FBS + 0.1 mM 2-ME / DMEM（low glucose）
*** 初期分化であれば10日間程度の培養により様々な分化マーカーが発現していくる。十分な分化を見るためには、一般的には 1 ヶ月から 3 ヶ月間培養が必要だと言われている。

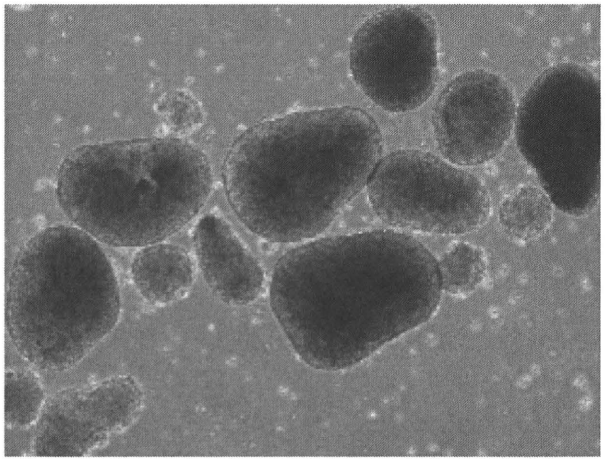

図1：京都大学樹立ヒト ES 細胞 KhES-1 の EB（40倍）

レート（AggreWell400、ベリタス）などが各種開発されている。簡単な方法としては、トリプシン／EDTA により細胞をシングルセルに分散させ、細胞密度を調整し、ROCK インヒビターを用いて細胞を播種する。細胞密度が高ければ大きな EB となり、細胞密度が低ければ、小さな EB となる。

(4) EB 作成による分化能の評価

EB を作成後、1 週間から 3 ヶ月間培養を行ったのち、RNA を抽出して RT-QPCR により遺伝子発現を解析する。遺伝子をリストアップして発現解析を行うか、あるいは、PCR アレイ、あるいはマイクロアレイ解析などが行われる[9]。ISCI の報告によれば、未分化状態のES細胞であっても分化マーカーと認識されている遺伝子発現も見られる。実際のヒト ES/iPS 細胞の維持培養においては、すべての細胞が未分化状態を示しているわけではなく、分化能を有した株であれば、様々な細胞に分化した細胞が混在する。また、未分化性に関与が

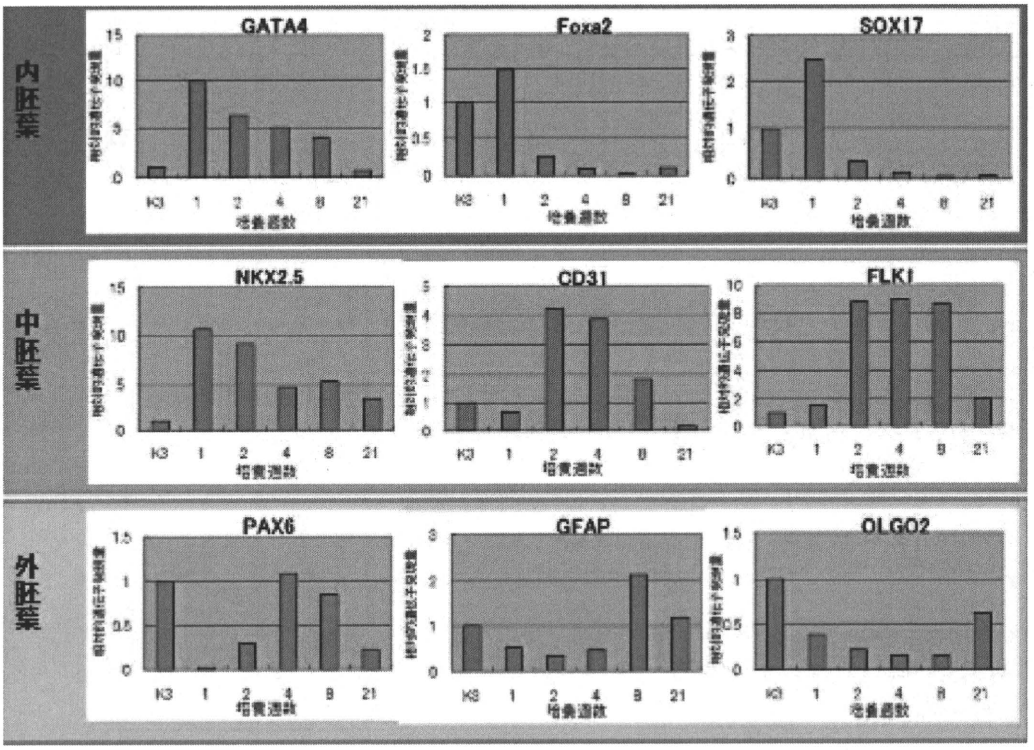

図２：EB の時間経過的遺伝子発現
成育医療センター梅澤ら樹立ヒト iPS 細胞 JCRB1331（Tic）の EB における経時的遺伝子発現を解析した。表１の手順により EB を形成させ、培養１週間、２週間、４週間、８週間、21週間後に RNA を抽出し、RT-QPCR により遺伝子発現解析を行った。他のクローンと比較することを目的としているために、京都大学樹立ヒト ES 細胞 KhES-3 の未分化状態の遺伝子発現を１として換算している（平田、松村、林田、その他、論文投稿準備中）。

解明されていない遺伝子の発現なども考えられ、必ずしも期待する分化マーカーの発現が、未分化状態より EB の方が高いとは限らない。プレリミナリーなデータとなるが、経時的な JCRB1331（Tic）の EB における遺伝子発現をみると、培養期間が１週間から２ヶ月まではそれぞれの遺伝子発現の増減が見られる（図２）。三胚葉の遺伝子を単純に選んだだけでは、分化能を評価することができないことがわかる。また、遺伝子を解析する時期も重要であることが示唆される。これらの遺伝子の発現の増減が株間でどのくらいの差があるのか、現在、検討中である。

(5) そのほかの分化能評価法

ヒト ES/iPS 細胞をヌードマウスや SCID マウスなどに移植をして、テラトーマ形成をさせるのがもっとも簡単で正確な評価法であると言われる。しかし、技術的な問題や細胞の生着率の効率もあり、正確に分化能を反映していると断言するのは難しい。また、EU においては、動物愛護の観点から動物実験の許可が難しく、テラトーマ形成能を ES/iPS 細胞の分化能評価として利用されることは多くない。これらのことから、ヒト ES/iPS 細胞の分化能の評価は、テラトーマ形成を必須であるとまではされていない。

謝　　辞

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Standardization of human embryonic stem (ES) cell and induced pulripoent stem (iPS) cell research in Japan: How to detect differentiation potency of human ES/iPS cells

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Abstract　In 1998, human embryonic stem (hES) cells have been established. In 2007, human induced pluripotent stem (hiPS) cells have been developed. Since then, the hES/iPS cells have been used as a tool for understanding the mechanisms in human development and regeneration application research in the world. hES cells are retrieved from the inner cell mass of human blastocyts. Therefore, it has been considered that hES cells should posses the possibility to differentiate into all cell types in human body. hiPS cells might have the similar characteristics to those of hES cells. To develop hES/iPS cell lines, it should be important to determine the pluripotency of the cells. In this review, I have summarized the differentiation potency of human ES and iPS cells.

Key Words　human ES cells, human iPS cells, embryoid body, differentiation

Genes to Cells

Efficient reprogramming of human and mouse primary extra-embryonic cells to pluripotent stem cells

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Practical clinical applications for current induced pluripotent stem cell (iPSC) technologies are hindered by very low generation efficiencies. Here, we demonstrate that newborn human (h) and mouse (m) extra-embryonic amnion (AM) and yolk-sac (YS) cells, in which endogenous *KLF4/Klf4*, *c-MYC/c-Myc* and *RONIN/Ronin* are expressed, can be reprogrammed to hiPSCs and miPSCs with efficiencies for AM cells of 0.02% and 0.1%, respectively. Both hiPSC and miPSCs are indistinguishable from embryonic stem cells in colony morphology, expression of pluripotency markers, global gene expression profile, DNA methylation status of *OCT4* and *NANOG*, teratoma formation and, in the case of miPSCs, generation of germline transmissible chimeric mice. As copious amounts of human AM cells can be collected without invasion, and stored long term by conventional means without requirement for in vitro culture, they represent an ideal source for cell banking and subsequent 'on demand' generation of hiPSCs for personal regenerative and pharmaceutical applications.

Introduction

Induced pluripotent stem cells (iPSCs) have been generated through nuclear reprogramming of somatic cells via retrovirus or lentivirus-mediated transduction of exogenous reprogramming factors Oct4, Sox2, Klf4 and C-Myc (Yamanaka 2007). This has led to greatly enhanced promise for exploring the causes of, and potential cures for, many genetic diseases, as well as increased promise for regenerative medicine. Improvements in delivery methodology have further facilitated iPSC generation by minimizing the requirement for genetic modification (Feng *et al.* 2009). Notably, generation of genetic modification-free iPSCs with reprogramming proteins (Kim *et al.* 2009; Zhou *et al.* 2009) suggests regenerative medicine with personal iPSCs could soon be realized. However, the markedly low efficiency of iPSC generation, with all adult somatic cell types tested to date, remains problematic (Wernig *et al.* 2008). Technological advancements in this field have mainly been achieved using mouse embryonic fibroblasts (MEFs), in which the efficiency of iPSC generation is 10–100 times higher than that with adult somatic cells (Yu *et al.* 2007; Wernig *et al.* 2008). Therefore, current methods would appear to be less than ideal for generating iPSCs from adult somatic cells.

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Here, to find nuclear reprogramming-sensitive cells collectable with no risk by physical invasion, we generated iPSCs from human and mouse newborn extra-embryonic membranes, amnion (AM) and yolk sac (YS), which consist huge amounts of discarded cells after birth. Interestingly, the efficiency of mouse iPSC (miPSC) generation from the AM was comparable to that of MEFs by retroviral transduction with *Oct4*, *Sox2*, *Klf4* and *c-Myc*. Importantly, human iPSC (hiPSC) is also efficiently generated from human AM cells. Expression of the endogenous *KLF4/Klf4*, *c-MYC/c-Myc* and *RONIN/Ronin* in human/mouse AM cells may function in facilitating the generation efficiency of iPSCs. The human AM cell, which is conventionally freeze-storable, could be a useful cell source for the generation of pluripotent stem cells including iPSCs mediated by nuclear reprogramming in the purpose of personal regenerative and pharmaceutical cure in the future of infants.

Results

Generation of iPSCs from mouse AM and YS cells

Extra-embryonic membranes, AM (amniotic ectoderm and mesoderm layers) and YS (visceral yolk sac endoderm and mesoderm layers) express a high level of proto-oncogene (Curran *et al.* 1984) which function, at least in part, to maintain and protect the fetus in utero. In E18.5 mouse embryos just before birth, AM and YS can be easily recognized microscopically (Fig. 1a). The membranes were dissected from *Oct4-GFP* (OG)/*Neo-LacZ* (Rosa26) embryos as approximately 5–10 mm^2 sections and digested with collagenase. Isolated cells were cultured for 4–5 days resulting in morphologically heterogeneous populations (Fig. 1a) in which OG expression was undetectable. Approximately 1×10^5 cells were then retrovirally transfected with exogenous Oct4, Sox2, Klf4 and c-Myc (OSKM). After approximately 3 weeks, OG-positive embryonic stem cell (ESC)-like miPSC colonies were picked and expanded without drug selection. All AM (female) and YS (male)-miPSC lines generated here, which closely resembled ESCs in morphology (Fig. 1a), had a $2n = 40$ normal karyotype (data not shown).

Characterization of AM and YS-miPSCs

As with ESCs, all AM- and YS-miPSC colonies were positive for alkaline phosphatase (ALP) (Fig. 1b). Immunohistochemical analyses also demonstrated that the cells were positive for pluripotent cell-specific nuclear proteins Oct4 and Nanog, and the surface glycoprotein SSEA1 (Fig. 1b). Thus, the expression profile of all marker proteins tested in AM and YS-miPSCs was similar to that observed in ESCs.

To examine the global transcription profile of these cells, comparative Affymetrix gene expression microarray analyses were performed between AM cells, YS cells, YS-miPSCs and R1 ESCs (Fig 1c). The global gene expression profile of YS-miPSCs was significantly different from that of YS cells. We detected a similar behavior between AM-miPSCs and AM cells (data not shown). Notably, the profile was similar to that of ESCs (Fig. 1c). Together, the data indicate that significant global nuclear reprogramming had occurred in these cells in response to OSKM transfection. We next applied RT-PCR analysis to gain a more focused transcriptional profile of pluripotent cell-specific marker genes in the induced cells. We found that *Nanog*, *Rex1*, *ERas*, *Gdf3*, *Zfp296* and *Ronin* were expressed in both AM and YS-miPSCs, whereas the AM and YS genes, *Igf1* and *Cd6* were silenced (Fig. 1c). Notably, *Ronin* was expressed not only in AM and YS-miPSCs but also in the precursor AM and YS cells. To investigate whether the exogenous *Oct4*, *Sox2*, *Klf4* and *c-Myc* genes were silenced by DNA methylation as reported for other iPSCs (Jaenisch & Young 2008) in the AM and YS-miPSCs, we examined expression using gene-specific primer sets designed to distinguish endogenous and exogenous transcripts. In all miPSC lines, the expression of endogenous *Oct4*, *Sox2*, *Klf4* and *c-Myc* was similar to that in R1 ESCs, whereas the exogenous *c-Myc* and *Klf4* were fully silenced in some YS-miPSC clones but not in others (Fig. 1c). Notably, high-level expression of endogenous *Klf4* and *c-Myc* was detected even in AM and YS cells, consistent with the expression of proto-oncogene (Curran *et al.* 1984). Endogenous expression of *Klf4*, *c-Myc* and *Ronin* genes that are involved in maintaining pluripotency may play a key function in enhancing the generation efficiency of miPSCs from AM and YS cells.

Timing and efficiency of miPSC generation

The molecular mechanisms that govern OKSM-induced nuclear reprogramming of somatic cells to iPSCs are poorly understood. It has been demonstrated that activation of endogenous *Oct4* may be a landmark for irreversible epigenetic transition toward

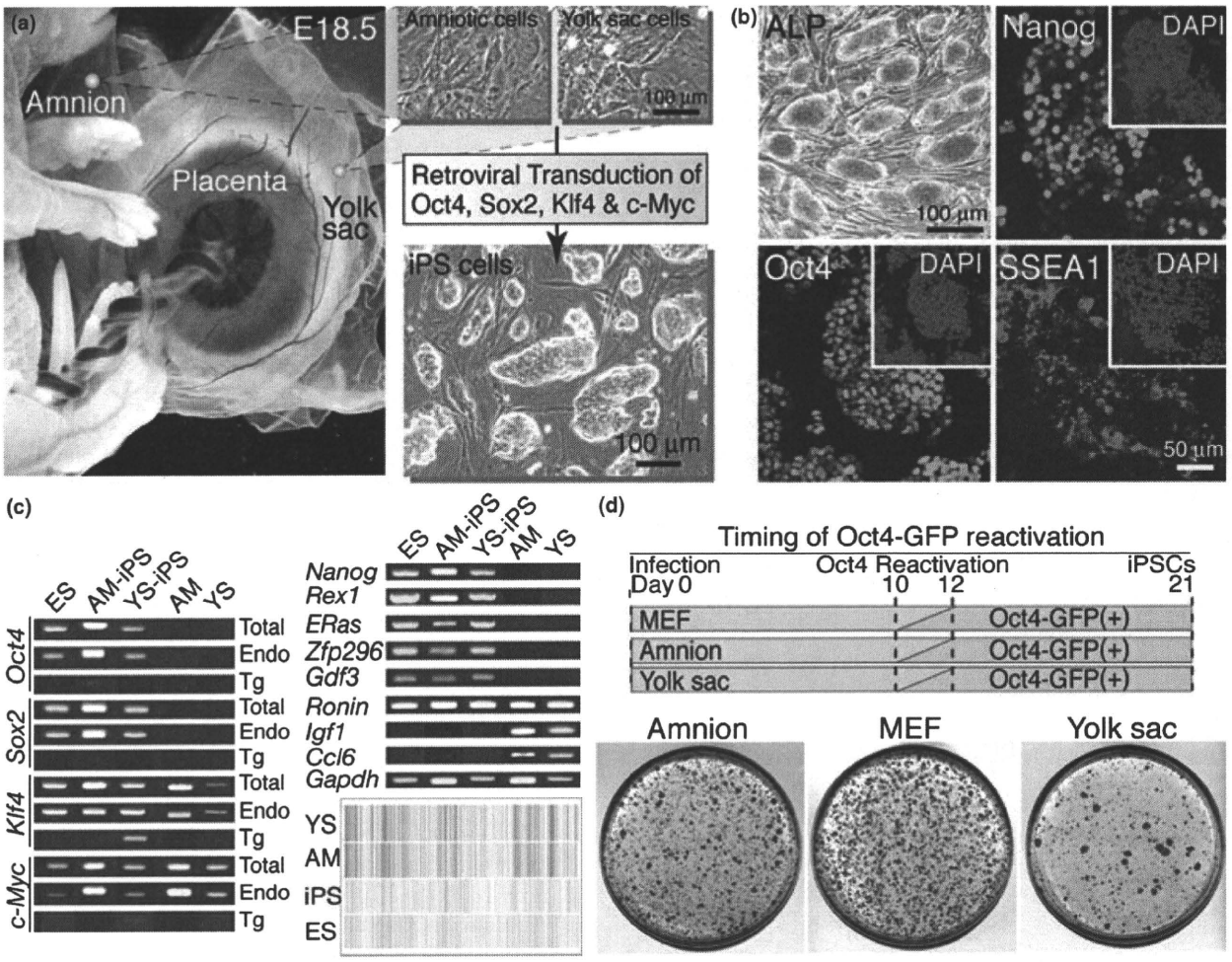

Figure 1 Generation of iPSCs from mouse AM and YS cells. (a) Isolation of AM and YS cells from the extra-embryonic tissues of newborn mice and generation of miPSCs through epigenetic reprogramming by retroviral infection-mediated expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc*. (b) Expression of pluripotent cell marker proteins, alkaline phosphatase (ALP), Nanog, Oct4 and SSEA1. Cell nuclei were visualized with DAPI. (c) Transcriptional activation and silencing of pluripotent and somatic cell marker genes by miPSC induction. RT-PCR analyses revealed that pluripotent marker genes were activated, somatic marker genes were silenced, and *Klf4*, *c-Myc* and *Ronin* were expressed even in AM and YS cells. *Gapdh* is a positive control. Microarray analyses demonstrated global alteration in gene expression profile between YS cells and YS-miPSCs, which more closely resemble mESCs. Relative level of gene expression is illustrated as red > yellow > green. (d) The generation efficiency of ALP-positive colonies and timing of GFP detection demonstrating *Oct4-GFP* reporter gene reactivation. ALP-positive colonies (red) in a 10-cm culture dish was shown when 1.0×10^5 of AM cells, YS cells and MEFs were exposed to OSKM reprogramming factors and reseeded at day 4.

fully reprogrammed iPSCs (Sridharan & Plath 2008). Thus, the timing of reactivation of OG is closely linked with the efficiency of reprogramming. Activation of exogenous OG was detected in some cell populations in every colony around 10 days after OSKM transfection of AM and YS cells, similar to control MEFs examined here and those reported previously (Fig. 1d) (Brambrink *et al.* 2008). The reprogramming efficiency of AM and YS cells was estimated by ALP-staining 21 days after OSKM transfection with reseeding at day 4. Notably, the number of ALP-positive colonies was similar between AM cells (4373 ± 983; mean ± SEM, $n = 3$) and MEFs (4997 ± 1049, $n = 3$), and ~50% in YS cells (2293 ± 487, $n = 3$). Thus, the efficiency of AM reprogramming by OSKM is comparable to that of MEFs, and far exceeds that of adult somatic cells (Fig. 1d).

Germline-transmissible chimeras with AM and YS-miPSCs

To address *in vivo* differentiation potential of the AM and YS-miPSCs, approximately 10 agouti miPSCs were microinjected into C57BL/6J × BDF1 blastocysts (black), and transferred into white ICR foster mothers to generate chimeras. Three male YS-miPSC and two female AM-miPSC lines were tested for chimera formation. X-gal staining analysis on sections of E15.5 embryos demonstrated successful generation of normally developing chimeric embryos with OG/*Neo-LacZ* miPSC contribution to the majority of tissues in all miPSC lines examined (data not shown). We next examined the miPSC potential for normal growth to sexual maturity and germline transmission. Two high-degree chimeric mice with a YS-miPSC line and three high-degree chimeric mice with two AM-miPSC lines, characterized by the >50% contribution of agouti coat color (Fig. 2a), developed normally into adulthood. However, an adult YS-miPSC chimera developed a neck tumor around 8–10 weeks after birth, which may be due to reactivation of the exogenous *c-Myc* as reported previously (Nakagawa *et al.* 2008). Testes isolated from affected males were bisected and one-half was X-gal-stained for LacZ activity whereas the other half was cryosectioned. Blue staining in the seminiferous tubule indicated that YS-miPSCs could contribute to germ cell development. To confirm this, testis cryosections immunohistochemically stained with antibodies against LacZ (iPSC-derived cell marker) and TRA98 (spermatogonia and spermatocyte marker) (Fig. 2b). Germ cells in all tubules were positive for TRA98, whereas germ cells in only some seminiferous tubules were positive for LacZ, clearly demonstrating that YS-miPSCs are capable of contributing to the differentiating germ line in chimeras. Finally, to examine whether the genetic information of YS-miPSCs was transmissible to the next generation, DNA isolated from progeny of the remaining YS-miPSC chimera was analyzed by genomic PCR with a primer set specific to *Neo*. Seven of the thirty-five pups examined were positive, demonstrating that YS-miPSCs are able to differentiate into fully functional germ cells (Fig. 2c). In one of three female AM-miPSC chimeric mice, competence for contribution to germ cells was detected by X-gal staining analysis of ovaries (data not shown).

Teratoma formation with AM and YS-miPSCs

The differentiation competence of AM and YS-miPSCs was further tested by teratoma formation induced by injection of cells into the inguinal region of immunodeficient SCID mice. Teratomas were isolated 5–8 weeks after for histological analysis and for gene expression analysis. Hematoxylin–eosin (HE) staining of paraffin sections demonstrated that the three primary layers were generated as morphologically shown by ectodermal glia and neuroepithelium, mesodermal muscle and endodermal ciliated epithelium and cartilage (Fig. 2d). Multi-lineage differentiation of miPSCs was verified by transcription of endodermal, mesodermal and ectodermal genes in the majority of teratomas (Fig. 2e).

Generation of iPSCs from human AM cells

To examine whether hiPSCs could be efficiently generated from primary AM cells isolated from the amniotic membrane (~100 cm^2) of the placenta of newborn human (Fig. 3a), the reprogramming factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* were introduced by vesicular stomatitis virus G glycoprotein (VSV-G) retroviral transduction. About 20 AM-hiPSC lines were established from 1.0×10^5 AM cells infected (0.02%). The efficiency of AM-hiPSC generation is markedly high relative to that with cells from human adult tissues (Yu *et al.* 2007). AM-hiPSCs were morphologically similar to human ESCs (hESCs) (Fig. 3a). Immunohistochemical analyses demonstrated expression of the pluripotent cell-specific nuclear proteins OCT4, SOX2 and NANOG, and the keratan sulfate proteoglycan TRA-1-60 (Fig. 3b) consistent with the profile observed in hESCs. To extend this analysis, we examined the expression profile of genes by RT-PCR. The endogenous reprogramming factor genes *OCT4*, *SOX2*, *KLF4* and *c-MYC* were all activated in AM-hiPSCs, whereas the transgenes were fully silenced (Fig. 3c). Expression of pluripotent cell-specific genes *NANOG, REX1, GDF3, ESG1, FGF4, TERT* and *RONIN* were also activated in all AM-hiPSC clones consistent with the profile of control hESCs (Fig. 3c). Notably, transcription of *KLF4, c-MYC,* and *RONIN* was detected not only in AM-hiPSCs but also AM cells. Similar to mouse AM and YS cells, endogenous expression of *KLF4, c-MYC* and *RONIN* in human AM cells may facilitate acquisition of reprogramming competency for efficient generation of hiPSCs.

DNA methylation of OCT4 and NANOG in AM-hiPSCs

To further characterize the pluripotent nature of AM-hiPSCs, the promoter CpG methylation status

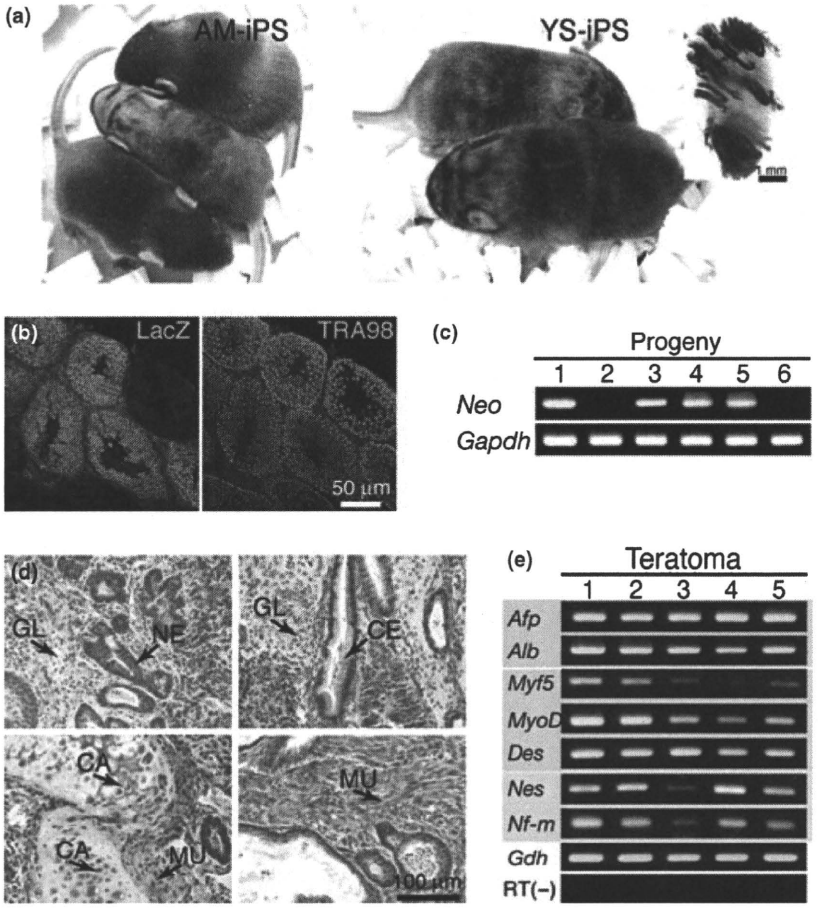

Figure 2 Pluripotency of AM and YS-miPSCs. (a) Chimeric mice with female AM-miPSCs and male YS-miPSCs. Inset: X-gal staining of testis collected from an adult YS-miPSC chimera (blue cells are YS-miPSC derivatives). (b) Immunohistochemical double staining of testis cryosections from a YS-miPSC chimera with anti-LacZ (YS-miPSC-derived germ cells) and anti-TRA98 (spermatogonia and spermatocytes) antibodies. (c) Genotyping of progeny obtained by backcrossing with YS-miPSC chimeras. *Neo* positive demonstrates germline transmission of YS-miPSC genetic information. *Gapdh* is positive control. (d) Hematoxylin-eosin staining of teratoma sections generated by AM and YS-miPSC implantation. GL, glia (ectoderm); NE, neuroepithelium (ectoderm); CE, ciliated epithelium (endoderm); CA, cartilage (ectoderm); MU, muscle (mesoderm). (e) Transcription analysis of lineage-specific genes in teratomas generated with AM and YS-miPSCs. Gray rectangle: endoderm makers; purple rectangle: mesoderm markers; pink rectangle: ectoderm markers. *Afp*, *α-Fetoprotein*; *Alb*, *albumin*; *Des*, *desmin*; *Nes*, *Nestin*; *Nf-m*, *neurofilament-M*; *Gdh*, *Gapdh* (positive control).

of key pluripotency genes was examined by bisulfite-modified DNA sequencing. Promoters of both *OCT4* and *NANOG* were found to highly methylated in hAM cells, consistent with transcriptional silencing in these cells. Conversely, both promoter regions were hypo-methylated in AM-hiPSCs consistent with the observed reactivation (Fig. 3d). These data demonstrate that human AM cells are capable of being epigenetically reprogrammed to AM-hiPSCs through forced expression of reprogramming factors.

Teratoma formation with AM-hiPSCs

To address whether the AM-hiPSCs have competence to differentiate into specific tissues, teratoma formation was induced by implantation under the kidney capsule of immunodeficient nude mice. Twenty-one out of twenty-four AM-hiPS independent clones induced teratoma formation within 6–10 weeks of implantation (1.0×10^7 cells/site). Histological analysis by HE staining of paraffin-embedded sections demonstrated that the three

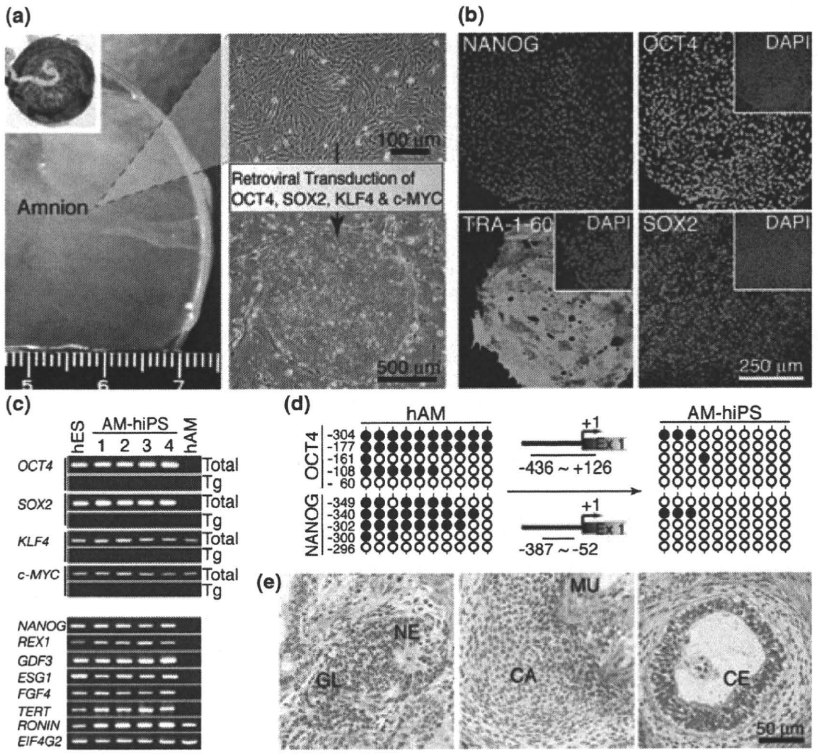

Figure 3 Generation of iPSCs from human AM cells. (a) Isolation of hAM cells from extra-embryonic tissues of human newborns and generation of hiPSCs through epigenetic reprogramming by retroviral infection-mediated expression of *OCT4*, *SOX2*, *KLF4* and *c-MYC*. (b) Expression of pluripotent cell marker proteins, NANOG, OCT4, TRA-1-60 and SOX2. Cell nuclei were visualized with DAPI. (c) Transcriptional activation of pluripotent marker genes by hiPSC induction. RT-PCR analyses revealed that the exogenous *OCT4*, *SOX2*, *KLF4* and *c-MYC* genes were silenced and the endogenous pluripotent marker genes were activated in AM-hiPSCs. *KLF4*, *c-MYC* and *RONIN* were expressed even in hAM cells before reprogramming. *EIF4G2* (*eukaryotic translation initiation factor 4 gamma 2*) is included as a positive control. (d) Epigenetic reprogramming of the *OCT4* and *NANOG* promoter regions. Bisulfite-modified DNA sequence analysis demonstrated a transition from hyper-methylation in AM cells (black circles) to hypo-methylation in AM-hiPSCs (white circles). (e) Hematoxylin-eosin staining of teratoma sections of teratoma generated by AM-hiPSC implantation. GL, glia (ectoderm); NE, neuroepithelium (ectoderm); CE, ciliated epithelium (endoderm); CA, cartilage (ectoderm); MU, muscle (mesoderm).

primary layers were generated as shown by ectodermal glia and neuroepithelium, mesodermal muscle and endodermal ciliated epithelium and cartilage morphologically (Fig. 3e). Thus, the majority of AM-hiPSC clones have potential for multi-lineage differentiation *in vivo*.

Discussion

We here demonstrated that hiPSCs and miPSCs were efficiently generated from newborn AM cells, in which endogenous *Klf4*, *c-Myc* and *Ronin* were highly expressed. The generation efficiency of miPSCs from AM cells was comparable to that from MEFs in mice and was notably high to that from adult somatic cells in humans. The properties of AM-hiPSCs and AM or YS-miPSCs resemble those of fully reprogrammed iPSCs from other tissues and ESCs.

iPSCs are generated through epigenetic reprogramming of somatic cells. Information on the base sequence of DNA in nuclei is unchanged through the reprogramming, although the gene expression profile is altered through the reprogramming from the somatic cell to the iPSC type. Developmentally rewound iPSCs retain aged DNA base sequence information inherited from somatic cells. The base sequence of DNA accumulates mutations through aging with cell division and mis-repair. Young somatic cells are suitable for iPSC generation rather than aged somatic cells. Therefore, it is suggested that the AM cells accumulating less genetic mutation are safer than the adult somatic cells as a cell source for iPSC generation.

The generation efficiency of OG-positive colonies was approximately four times lower than that of ALP-positive colonies and it is likely that miPSC generation will be further reduced (Wernig *et al.* 2008). Furthermore, when pre-iPSCs are reseeded, the generation efficiency of iPSC outcome could be roughly estimated as $1/2^X$ (X = reseeded day after infection or transfection; doubling time of pre-iPSC is estimated as 24 h). Recently, iPSC generation technology has been developed and improved with MEFs and human embryonic or newborn fibroblasts (HNFs) as representative somatic cells. Even with these types of cells, application of the current technology resulted in a marked decrease in iPSC generation efficiency. The retroviral transduction-mediated miPSC generation efficiency is 0.05–0.1% with MEFs (Takahashi *et al.* 2007; Wernig *et al.* 2007). The generation efficiency of hiPSCs (~0.01% in ALP-positive colony and 0.0025% in hiPSC outcome) (Yu *et al.* 2007; Wernig *et al.* 2008) is ~10 times lower than that of miPSCs. The generation efficiency of genetic modification-free hiPSCs from HNFs by direct delivery of reprogramming proteins is estimated at about 0.001% in outcome (Kim *et al.* 2009). Notably, it is evident that the generation of hiPSCs from adult somatic cells is much harder than that from MEFs. In fact, analysis with a secondary dox-inducible transgene system shows that the efficiency varies between different somatic cell types (Wernig *et al.* 2008). Thus, for practical application of iPSC technology to medical care, identification of reprogramming-sensitive cell types is a key issue. Human primary keratinocytes are one candidate cell type for efficient generation of hiPSCs from adult patients (the efficiency of ALP-positive colony = 1.0%) (Aasen *et al.* 2008). Here, we have shown that human and mouse AM cells, in which the endogenous *KLF4/Klf4*, *c-MYC/c-Myc* and *RONIN/Ronin* are naturally expressed, are highly reprogramming-sensitive (hiPSC generation efficiency was approximately 0.02% in outcome). An important point is that relatively huge amounts of human AM cells can be collected from discarded AM membranes at birth with no risk to the individual. Furthermore, these cells can be kept in long-term storage without requirement for amplification by in vitro cell culture.

Our findings illustrate that human AM cells are a strong candidate cell source for collection and banking that could be retrieved on demand and used for generating personalized genetic modification-free iPSCs applicable for clinical treatment and drug screening.

Experimental procedures

Amnion and yolk sac cells

In mice, AM and YS membranes collected from E18.5 embryos from GOF-18/delta PE/GFP (Oct4-GFP) transgenic females (Yoshimizu *et al.* 1999) mated with 129/Rosa26 transgenic males (Friedrich & Soriano 1991) were digested with 0.1% collagenase (Wako, Osaka, Japan) and 20% fetal bovine serum (FBS) at 37 °C for 1 h, and then repeatedly passed through a 26-gauge needle. The cell suspension was cultured with mES medium (DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12) (Wako) supplemented with 15% FBS, 10^{-4}M 2-mercaptoethanol (Sigma) and 1000 U/mL of recombinant leukemia inhibitory factor (Chemicon, Temecula, CA, USA) containing 5 ng/mL basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA). Following culture for 2–3 days, the adherent AM and YS cells growing to near-confluence were applied for iPSC experiments.

In humans, the AM membrane was cut into tiny pieces with dissection scissors. The AM membrane pieces were cultured in DMEM with 10% FBS for 7–10 days. The adherent AM cells growing to near-confluence were applied for iPSC experiments. Primary AM cells were provided from the cell bank of RIKEN Bioresource Center, Japan.

Generation of iPSCs

In mouse, each of pMXs-Oct4, Sox2, Klf4, c-Myc and DsRed (an indicator of retroviral silencing) was transfected into the Plat-E cells using the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). A 1 : 1 : 1 : 1 : 4 mixture of Oct4, Sox2, Klf4, c-Myc and DsRed retroviruses in supernatants with 4 μg/mL polybrene (Nacalai Tesque, Kyoto, Japan) was added to AM and YS cells at 1.0×10^5 cells per 3 cm well. At day 4 after infection, the cells were reseeded into a 10 cm culture dish on feeder cells with mES medium. Colonies were picked around day 20.

In humans, pMXs-OCT4, SOX2, KLF4 or c-MYC, pCL-GagPol, and pHCMV-VSV-G vectors were transfected into 293FT cells (Invitrogen, Carlsbad, CA, USA) using the TransIT-293 reagent (Mirus). A 1 : 1 : 1 : 1 mixture of OCT4, SOX2, KLF4 and c-MYC viruses in supernatant with 4 μg/mL polybrene were added to AM cells at 1.0×10^5 cells per 3 cm well. The cells were subcultured on feeder cells into a 10 cm dish with the iPSellon medium (Cardio) supplemented with 10 ng/mL bFGF (Wako) (hES medium). Colonies were picked up around day 28.

Immunocytochemistry

Human and mouse cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at 4 °C. After washing with 0.1% Triton X-100 in PBS (PBST), the cells were prehybridized with blocking buffer for 1–12 h at 4 °C and then incubated with primary antibodies; anti-SSEA4

Table 1 Primers for RT-PCR and PCR

Gene name	5′-Forward-3′	5′-Reverse-3′
Mice		
Oct4 (total)	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT
Oct4 (endogenous)	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
Oct4 (transgene)	CCCATGGTGGTGGTACGGGAATTC	AGTTGCTTTCCACTCGTGCT
Sox2 (total)	GGTTACCTCTTCCTCCCACTCCAG	TCACATGTGCGACAGGGGCAG
Sox2 (transgene)	CCCATGGTGGTGGTACGGGAATTC	TCTCGGTCTCGGACAAAAGT
Klf4 (total)	CACCATGGACCCGGGCGTGGCTGCCAGAAA	TTAGGCTGTTCTTTTCCGGGGCCACGA
Klf4 (endogenous)	GCGAACTCACACAGGCGAGAAACC	TCGCTTCCTCTTCCTCCGACACA
Klf4 (transgene)	CCCATGGTGGTGGTACGGGAATTC	GTCGTTGAACTCCTCGGTCT
c-Myc (total)	CAGAGGAGGAACGAGCTGAAGCGC	TTATGCACCAGAGTTTCGAAGCTGTTCG
c-Myc (endogenous)	CAGAGGAGGAACGAGCTGAAGCGC	AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC
c-Myc (transgene)	CTCCTGGCAAAAGGTCAGAG	GACATGGCCTGCCCGGTTATTATT
Nanog	ATGAAGTGCAAGCGGTGGCAGAAA	CCTGGTGGAGTCACAGAGTAGTTC
Eras	CAAAGATGCTGGCAGGCAGCTACC	GACAAGCAGGGCAAAGGCTTCCTC
Gdf3	AGTTTCTGGGATTAGAGAAAGC	GGGCCATGGTCAACTTTGCCT
Rex1	GACATCATGAATGAACAAAAAATG	CCTTCAGCATTTCTTCCCTG
Zfp296	AAGCACCCAGATCTGTTGACCT	GAGCCTCTGGGGTATCTAGG
Ronin	GCCTCAGAGCTAGAGGCTGCTACG	TGGAAGGAGTCACGAATTCTGCAG
Igf1	GGACCAGAGACCCTTTGCGGGG	GGCTGCTTTTGTAGGCTTCAGTGG
Cd6	CCTAAGCACCCTGAAGCAAG	ACAACTGGGAACCCACAAAGC
Gapdh	CCCACTAACATCAAATGGGG	CCTTCCACAATGCCAAAGTT
α-Fetoprotein	TCGTATTCCAACAGGAGG	CACTCTTCCTTCTGGAGATG
Albumin	AAGGAGTGCTGCCATGGTGA	CCTAGGTTTCTTGCAGCCTC
Myf-5	TGCCATCCGCTACATTGAGAG	CCGGGTAGCAGGCTGTGAGTTG
MyoD	GCCCGCGCTCCAACTGCTCTGAT	CCTACGGTGGTGCGCCCTCTGC
Desmin	TTGGGGTCGCTGCGGTCTAGCC	GGTCGTCTATCAGGTTGTCACG
Nestin	GGAGTGTCGCTTAGAGGTGC	TCCAGAAAGCCAAGAGAAGC
Neurofilament-M	GCCGAGCAGACCAAGGAGGCCATT	CTGGATGGTGTCCTGGTAGCTGCT
Neo	CGGCAGGAGCAAGGTGAGAT	CAAGATGGATTGCACGCAGG
Humans		
OCT4 (total)	GCCGTATGAGTTCTGTGG	TCTCCTTCTCCAGCTTCAC
SOX2 (total)	TAAGTACTGGCGAACCATCT	AAATTACCAACGGTGTCAAC
KLF4 (total)	ACTCGCCTTGCTGATTGTCT	GAACGTGGAGAAAGATGGGA
c-MYC (total)	GCGTCCTGGGAAGGGAGATCCGGAGC	TTGAGGGGCATCGTCGCGGGAGGCTG
NANOG	ATTATGCAGGCAACTCACTT	GATTCTTTACAGTCGGATGC
REX1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA
GDF3	CTTATGCTACGTAAAGGAGCGGG	GTGCCAACCCAGGTCCCGGAAGTT
ESG1	ATATCCCGCCGTGGGTGAAAGTTC	ACTCAGCCATGGACTGGAGCATCC
FGF4	CTACAACGCCTACGAGTCCTACA	GTTGCACCAGAAAAGTCAGAGTTG
TERT	CCTGCTCAAGCTGACTCGACACCGTG	GGAAAAGCTGGCCCTGGGGTGGAGC
RONIN	CACTGTAGACAGCAGTCAGG	TGCCTTTCATCTCTTTCATC
EIF4G2	AAGGAAAGGGACTGAGTTTC	CCAAGAAAGCTTCTTCTTCA
Bis-OCT4	GATTAGTTTGGGTAATATAGTAAGGT	ATCCCACCCACTAACCTTAACCTCTA
Bis-NANOG	TGGTTAGGTTGGTTTTAAATTTTTG	AACCCACCCTTATAAATTCTCAATTA

(1 : 300) (Chemicon), anti-TRA-1–60 (1 : 300) (Chemicon), anti-Oct4 (1 : 50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nanog (1 : 300) (ReproCELL, Tokyo, Japan), anti-Sox2 (1 : 300) (Abcam, Cambridge, UK) and/or anti-SSEA1 (1 : 1000) (DSHB) antibodies for 6–12 h at 4 °C. They were incubated with secondary antibodies; anti-rabbit IgG, anti-mouse IgG or anti-mouse IgM conjugated with Alexa 488 or 546 (1 : 500) (Molecular Probes, Eugene, OR, USA) in blocking buffer for 1 h at room temperature. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and then mounted with a SlowFade light antifade kit (Molecular Probes). To examine germline competence,

cryosections of a half of a testis of 4- to 5-week-old chimeric mice were fixed with 4% paraformaldehyde in PBS for overnight at 4 °C, and then prehybridized with blocking buffer. The sections were double-stained with primary antibodies; anti-LacZ antibody (1 : 500) (Promega, Madison, WI, USA) specific to miPSC-derived cells and with anti-TRA98 antibody (1 : 500) specific to spermatogonia and spermatocytes. The remaining testis and ovaries were stained with X-gal.

RT-PCR

Total RNAs were isolated from mouse and human cells using the TRIzol (Invitrogen) and the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), respectively. cDNAs were synthesized from 1 μg total RNAs using Superscript III reverse transcriptase (Invitrogen) with random hexamers according to the manufacturer's instructions. Template cDNA was PCR-amplified with gene-specific primer sets (Table 1).

Gene expression microarray

Total RNA was extracted from mouse cells using the TRIzol Reagent. Double-stranded cDNA synthesized from the total RNA was amplified and labeled using the One-Cycle Target Labeling and Control Regents (Affymetrix, Santa Clara, CA, USA). Global gene expression was examined with the GeneChip Mouse Genome 430 2.0 Array (Affymetrix). The fluorescence intensity of each probe was quantified by using the GeneChip Analysis Suite 5.0 computer program (Affymetrix). The level of gene expression was determined as the average difference (AD). Specific AD levels were then calculated as percentages of the mean AD level of probe sets for housekeeping genes *Actin* and *Gapdh*. To eliminate changes within the range of background noise and to select the most differentially expressed genes, data were used only if the raw data values were less than 50 AD. Further data were analyzed with GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA, USA).

Reprogramming efficiency

The reprogramming efficiency of mouse YS and AM cells was estimated by counting the number of ALP-positive colonies 21 days after retroviral infection. The cells in 10 cm culture dish were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS. After treating with ALP stain (pH 9.0) for 30 min at room temperature, the number of ALP-positive cells was counted.

Chimera

AM-miPSCs ($2n$ = 40, XX) and YS-miPSCs ($2n$ = 40, XY) were microinjected into blastocysts (C57BL/6J × BDF1). The blastocysts were transferred into the uterus of pseudopregnant ICR female mice. Chimeric mice were mated with C57BL/6J for examining germline transmission. The genotype of the progeny was determined with tail tip DNA by genomic PCR with a *Neo*-specific primer set (Table 1). All animal experiments were performed according to the guidelines of animal experiments of Kyoto University, Japan.

Teratoma

In mice, cell suspension of 1.0×10^6 AM or YS-miPSCs/100 μL DMEM/F12 was subcutaneously injected into the inguinal region of immunodeficient SCID mice (CLEA). In humans, the 1 : 1 mixture of the AM-hiPSC suspension and Basement Membrane Matrix (BD Biosciences, San Jose, CA, USA) were implanted at 1.0×10^7 cells/site under the kidney capsule of immunodeficient nude mice (CLEA). Teratomas surgically dissected out 5–8 weeks in mice and 6–10 weeks in human after implantation, were fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Sections at 10 μm in thickness were stained with HE.

Bisulfite-modified DNA sequencing

Genomic DNAs (1 μg) extracted from AM-hiPSCs and hAM cells were bisulfite-treated with EZ DNA methylation-Gold Kit (ZYMO Research, Orange, CA, USA) according to the manufacturer's instruction. The promoter regions of the human *NANOG* and *OCT4* genes were PCR-amplified with specific primer sets (Table 1). Ten clones of each PCR product were gel-purified, sub-cloned and sequenced with the SP6 universal primer.

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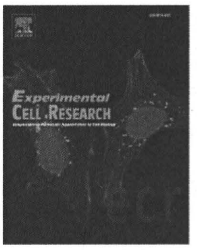

Research Article

Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POU5F1) with physiological co-activator EWS

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ABSTRACT

POU5F1 (more commonly known as OCT4/3) is one of the stem cell markers, and affects direction of differentiation in embryonic stem cells. To investigate whether cells of mesenchymal origin acquire embryonic phenotypes, we generated human cells of mesodermal origin with overexpression of the chimeric *OCT4/3* gene with physiological co-activator EWS (product of the *EWSR1* gene), which is driven by the potent *EWS* promoter by translocation. The cells expressed embryonic stem cell genes such as *NANOG*, lost mesenchymal phenotypes, and exhibited embryonal stem cell-like alveolar structures when implanted into the subcutaneous tissue of immunodeficient mice. Hierarchical analysis by microchip analysis and cell surface analysis revealed that the cells are subcategorized into the group of human embryonic stem cells and embryonal carcinoma cells. These results imply that cells of mesenchymal origin can be traced back to cells of embryonic phenotype by the *OCT4/3* gene in collaboration with the potent cis-regulatory element and the fused co-activator. The cells generated in this study with overexpression of chimeric OCT4/3 provide us with insight into cell plasticity involving OCT4/3 that is essential for embryonic cell maintenance, and the complexity required for changing cellular identity.

Introduction

Somatic stem cells have been shown to have a more flexible potential, but the conversion of mesenchymal cells to embryonic stem (ES) cells has still been a challenge and requires gene transduction [1–4]. This phenotypic conversion requires the molecular reprogramming of mesenchyme. Mesenchymal stem cells or mesenchymal progenitors have been isolated from adult bone marrow [5], adipose tissue [6], dermis [7], endometrium [8], menstrual blood [8], cord blood [9,10], and other connective tissues [11]. These cells are

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capable of differentiating into osteoblasts [12], chondrocytes [13], skeletal myocytes, adipocytes, cardiomyocytes [14,15], and neural cells [16]. However, most of the differentiation capability is limited to cells of mesodermal origin. This is in contrast to ES cells derived from the inner cell mass of the blastocyst that differentiate into cells of three germ cell layers. ES cells are pluripotent and immortal, and, therefore, ES cells provide an unlimited number of specialized cells.

Embryonic and adult fibroblasts have been induced to become pluripotent stem cells (iPS cells) or ES-like cells by defined factors including POU5F1 (also known as OCT4/3) [1–3]. OCT4/3 protein, a member of the POU family of transcription factors, is related to the pluripotent capacity of ES cells, and is thus a distinctive marker to identify primordial germ and embryonic stem cells [17–21]. OCT4/3 is down-regulated during oogenesis and spermatogenesis [22]. Furthermore, knocking out the *OCT4/3* gene in mice causes early lethality because of lack of inner cell mass formation [23], and OCT4/3 is critical for self-renewal of ES cells [24]. During human development, expression of OCT4/3 is found at least until the blastocyst stage [25] in which it is involved in gene expression regulation. OCT4/3 functions as a master switch in differentiation by regulating cells that have, or can develop, pluripotent potential by activating transcription via octamer motifs [26].

The EWS gene was originally identified at the chromosomal translocation, and fused with the ets transcription factors in Ewing sarcoma, as is the case of other sarcomas [27–30].

We report here the generation of human cells that overexpress the *OCT4/3* gene with physiological co-activator EWS (translation product of the *EWS* gene). In this study we show that the cells of mesenchymal origin overexpressing OCT4/3 can be traced back to cells with an embryonic phenotype.

Materials and methods

Cell culture

GBS6 cells were generated from primary or first passage cells of a pelvic tumor [31], and cultured in tissue culture dishes (100 mm, Becton Dickinson) in the G031101 medium (Med Shirotori, Tokyo). All cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. When the cultures reached subconfluence, the cells were harvested with Trypsin–EDTA Solution (cat# 23315, IBL) at 0.06% trypsin, and replated at a density of 5×10^5 in a 100 mm dish. Medium changes were carried out twice weekly thereafter. Both H4-1 and Yub10F were human bone marrow cells. The 3F0664 were human bone marrow-derived mesenchymal cells and were purchased from Lonza (PT-2501, Basel, Switzerland). The H4-1, Yub10F and 3F0664 cells were cultured in the mesenchymal-stem-cell-growth (MSCG)-Medium-BulletKit (PT-3001, Lonza). The NCR-G1 (a human yolk sac tumor line), NCR-G2 (a human embryonal carcinoma cell line from a testicular tumor), NCR-G3 (a human embryonal carcinoma cell line from a testicular tumor) and NCR-G4 (a human embryonal carcinoma cell line) were cultured in the G031101 medium as previously described [32]. In an experiment to inhibit cell adhesion, GBS6 and NCR-G3 cells were treated with anti-human E-cadherin, monoclonal (Clone HECD-1) (M106, TAKARA BIO INC.) at 100 μg/mL. Treatment with the demethylating agent, 5′-aza-2′-deoxycytidine (5azaC; A2385, SIGMA), was performed on GBS6 cells. GBS6 cells were treated with 3 μM of 5azaC for 24 h, and then cultured without treatment for 24 h. The 5azaC-traeated GBS6 cells were described as "GBS6-5azaC". MRC-5 human fetal lung fibroblasts were maintained in POWEREDBY10 medium (MED SHIROTORI CO., Ltd, Tokyo, Japan). We used these cells at between 17 and 25 PDs for the infection of the retroviral vectors. 293FT cells were maintained in DMEM containing 10% FBS, 1% penicillin and streptomycin. iPS cells were maintained in iPSellon medium (007001, Cardio) supplemented with 10 ng/mL recombinant human basic fibroblast growth factor (bFGF, WAKO, Japan). For passaging, iPS cells were washed once with PBS and then incubated with Dulbecco's Phosphate-Buffered Saline (14190-144, Invitrogen) containing 1 mg/mL Collagenase IV (17104-019, Invitrogen), 1 mM $CaCl_2$, 20% Knockout Serum Replacement (KSR) (10828-028, Invitrogen), and 0.05% Trypsin–EDTA Solution (23315, IBL) at 37 °C. When colonies at the edge of the dish started dissociating from the bottom, DMEM/F12/collangenase was removed. Cells were scraped and collected into 15 mL conical tubes. An appropriate volume of the medium was added, and the contents were transferred to a new dish on irradiated MEF feeder cells. The split ratio was routinely 1:3.

G-banding karyotypic analysis and spectral karyotyping (SKY) analysis

Metaphase spreads were prepared from cells treated with Colcemid (Karyo Max, Gibco Co. BRL, 100 ng/mL for 6 h). We performed a standard G-banding karyotypic analysis on at least 50 metaphase spreads for each population. SKY analysis was performed on metaphase-transduced cells according to the kit manufacturer's instruction (ASI, Carlsbad, CA) and a previously published method [33].

RT-PCR

The cDNAs were synthesized with an aliquot (5 μg) of each total RNA using Oligo-(dT)20 primer (18418-020, Invitrogen) and SuperScript III Reverse Transcriptase (18080-044, Invitrogen). Both the RNA strand of an RNA–DNA hybrid and single-stranded DNA were degraded by RNaseH (18021-071, Invitrogen). For the thermal cycle reactions, cDNA was amplified by T3 Thermocycler (Biometra, Goettingen, Germany) under the following reaction conditions: 30 cycles of a PCR (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) after an initial denaturation (94 °C for 1 min). Primer sets used for PCR reactions are described in Tables 1 and 2. As the same amount of cDNA template was used in all reactions, in comparison to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard, the expression levels were evaluated. The controls consisted of reactions without reverse transcriptase in the process of cDNA synthesis.

Table 1 – PCR primers to detect the chimeric *EWS-OCT4/3* gene and untranslocated *OCT4/3* gene.

Symbol	Name	Sequence
A	EWS exon6-F	5′ TTA GAC CGC AGG ATG GAA AC 3′
B	EWS ex6 intron-F	5′ GTG GGG TTC ACT AT 3′
C	POU5F1-1a-F	5′ GAT CCT CGG ACC TGG CTA AG 3′
D	POU5F1-2-F	5′ CTT GCT GCA GAA GTG GGT GGA GGA A 3′
E	POU5F1-1a-R	5′ TCA GGC TGA GAG GTC TCC AA 3′
F	POU5F1-3-R	5′ CTG CAG TGT GGG TTT CGG GCA 3′