発を目的としており、ヒト間葉系幹細胞の培養環境・培養技術の改良による長期安定培養法の開発、ヒト間葉系幹細胞の核型解析および染色体プロファイル解析による長期培養における品質評価法開発を行い、再生医療への適用の可能性と問題点を検討した。

B. 研究方法

（1）細胞培養

2 種のヒト臍帯血由来間葉系幹細胞、UCBTERT-21 (JCRB1107)および UCB408E6E7 TERT-33 (JCRB1110)ならびに 2 種のヒト骨髄由来間葉系幹細胞、UE6E7T-3 (JCRB1136)および UBE6T-6 (JCRB1140) は当 JCRB 細胞バンク(Osaka, Japan)で保存された細胞を使用した。臍帯血由来間葉系幹細胞株 UCBTERT-21 は hTERT 遺伝子のみで、UCB408E6E7TERT-33 は hTERT と HPV16E6/E7 の組み合わせ遺伝子により不死化したものであり、Plusoid M (Med-Shirotori Co. Japan) 培地で培養した。また、骨髄由来間葉系幹細胞株 UE6E7T-3 は hTERT と HPV16E6E7 遺伝子で、UBE6T-6 は hTERT、HPV16E6、および Bmi-1 遺伝子の組み合わせ導入により不死化したものであり、Poweredby 10 培地 (Med-Shirotori Co. Japan) で培養した。培養開始の細胞密度は 2000 cells/$cm^2$ で 37℃、5% CO2 の条件下で培養した。PDLs の計算は次の式に従った: PDLs = log (cell output/cell input)/log2。本実験で培養を開始したときの PDLs は、UCBTERT-21, UCB408E6E7TERT-33, UE6E7-3 と UBE6T-6 それぞれ 42, 67, 40 と 56 であった。同様にヒト間葉系幹細胞 hMSC (Cambrex 社より購入) に関しても、MSCBM (間葉系幹細胞培養用培地) と DMEM の 2 種類の培地で培養を行い経時的観察を行った。

（2）染色体解析

細胞は直径 100mm のプラスチックディシュで培養し、継代後約 2 日目にコルセミドを加え、37℃で 2 時間インキュベートした後、トリプシンで剥離し、デイッシュから回収した。次に 0.075M・KC l 低張処理後、カルノア液で固定した。染色体数の測定には metaphase spread chromosome を DAPI 染色し、Axioplan II imaging microscope (Carl Zeiss, GmbH) で観察し、プログラムソフト LeicaQFISH を用いて画像の取得と解析をした。pFISH 解析には 13 番染色体、17 番染色体に特異的なプローブ(XCP13 - kit - FITC、XCP17 - kit - Texas Red) (MetaSystems、GmbH)を、mFISH の解析にはマルチカラープローブ (24XCyte-MetaSystems' 24color kit) を用いた。方法は MetaSystems 社のプロトコールに従った。FISH 像は Zeiss Axio imaging microscope (Carl Zeiss Microimaging, GmbH) で観察し、プログラムソフト mBAND/mFISH (MetaSystems, GmbH) で解析した。

（3）CGH アレイ解析 (Macrogen 社)

サンプル DNA は約 $1\times10^6$ 個の細胞から isolation kit (Amersham BioSciences, UK) と Spin Column (QIAGEN Co., Japan)を用いて抽出・精製した。標準 DNA (Promega Co. USA) と試験 DNA は それぞれ Cy3 または Cy5 (BioPrimer DNA Labeling System, Invitrogen Co., Japan)で標識し、Cot-1 DNA とエタノールで共沈殿し、ハイブリダイゼーションキット(50% formamide, 10% dextran sulfate, 2xSSC, 4% sodium dodecyl sulfate (SDS), pH7)に溶かした。75℃ 10 分処理で DNA を変性したのち、BAC Array (MAC ArrayTM Karyo 4000 Component, Macrogen Co., USA) で 42℃、48-72 時間ハイブリダイゼーションした。50% formamide - 2x SSC (pH 7.0) で 50℃、15 分間, 2x SSC - 0.1% SDS で 50℃、15 分間洗浄し、100 mM sodium phosphate buffer (0.1% Nonidet P-40 (pH 8)を含む) で洗浄したのち各スポットの蛍光量を

GenePix4000A (Axon Instruments, USA)で測定し、MacViewer (Macrogen Instruments, USA)を用いてデータを解析した。

（4）CGHアレイ解析（アジレント社）

サンプルDNAは約5x$10^6$個の細胞からAllPrep DNA/RNA Mini kit (QIAGEN Co., Japan)を用いて抽出・精製した。2種類のDNA試料は、それぞれAlexa FluorR 3またはAlexa FluorR 5 (BioPrime® Total Genomic Labeling System, Invitrogen Co., Japan)で標識した。標識したDNAとCot-1 DNAをハイブリダイゼーション溶液(10 x Blocking Agent, 2 x Hybridization Buffer)に溶かした。95℃ 3分処理でDNAを変性したのち、37℃ 30分インキュベーションした。CGHマイクロアレイスライド(Human Genome CGH 244A Oligo Microarray kit , Agilent Co., Japan)にアプライし、65℃ 40時間ハイブリダイゼーションした。Agilent Oligo aCGH洗浄バッファ1で室温、5分間, Agilent Oligo aCGH洗浄バッファ2で37℃、1分間洗浄したのち各スポットの蛍光量をDNA Microarray Scanner (Agilent Co., Japan)で測定し、Agilent Feature Extraction (Agilent Co., Japan)を用いてデータを解析した。

（5）分化能の測定

脂肪細胞への分化能を測定するため、カバースリップの上に培養した各細胞を誘導培地（hMSC Differentiation BulletKit - Adipogenic ; PT-3004, Camblex BioScience Waltervillo, Inc. USA）、神経細胞への誘導にはNPMM Bullet kit (NPMMTM BulletKit (B3209, Camblex BioScience Waltervillo, Inc. USA)を用いた。骨芽脂肪への分化誘導には 0.1 μM dexamethasone (Sigma Chemical Co., USA), 50 μg/ml L-ascorbic acid (Sigma Chemical Co., USA)と10 mM β-glycerophosphate (Sigma Chemical Co., USA)をPlusoid-M培地 (Med-Shirotori Co., Tokyo, Japan)培地またはPoweredby10培地 (Med-Shirotori Co., Tokyo, Japan)に入れ、2-4週間培養した。phosphate-buffered saline (PBS)で洗浄後, 4% paraformaldehydeで固定した。

脂肪細胞はOil Red-O (Sigma Chemical Co., USA)染色し、骨芽細胞には0.25 mg/ml naphthol AS-BI phosphateおよび0.25 mg/ml Fast violet LB saltでalkaline phosphatase染色した。神経細胞の観察には、パラフォルムアルデヒドとメタノール固定したのち、anti-Ⅲβ tubulin抗体 (Sigma Chemical Co. USA)またはanti -neurofilament antibody NF-200 (Sigma Chemical Co., USA)とTexas Red-anti -mouse IgG抗体 (Southern Biotechnology Associates, Inc., USA)で免疫染色した。

## C. 研究結果

### C.1　ヒト間葉系幹細胞の長期培養過程での染色体数の変化

研究に用いた不死化間葉系幹細胞において染色体数の異常が観察され、特に長期培養するとその頻度が高くなった。培養開始直後は全ての細胞株の染色体数モードは 46 本(2n)であった。UCBTERT-21 細胞は PDL 133 にも及ぶ長期培養後でも染色体数は安定であったが、一方、UBE6T-6 および UE6E7T-3 細胞には染色体数の変化がみられた。PDL 62 の UE6E7T-3 は 90 %の細胞が 46 本の染色体をもっていたが、PDL 147 では染色体欠失により 44 本になった細胞が 43%観察された。UBE6T-6 細胞でも同様に染色体数の減少が見られた。UCB408E6E7TERT-33 細胞もまた染色体数が不安定で、4 倍体に近い細胞集団の増加が認められた。これらの結果から、UE6E7T-3, UBE6T-6 および UCB408E6E7TERT-33 細胞の染色体数は長期培養でかなり変動するが、UCBTERT-21 にはその傾向が見られないことが示された。

次に、染色体の不安定性を調べるために、FISH 法、CGH 法による解析を行った。PDL 52 の UCBTERT-21 細胞の mFISH 分析の結果は正常な核型を示した(Fig. 1A and B) 。UBE6T-6 細胞は 43-45 本の染色体数を示し、13, 16 and 19 番染色体の欠失が検出された(Fig. 1D and E)。UCB408E6E7TERT-33 細胞も不均一な染色体構成を示した。mFISH 分析の結果から UCBTERT-21 細胞以外は共通して 13 番染色体の欠失が示されたが、この結果は 13 と 17 番染色体のプローブを用いた pFISH 分析によっても確かめられた (Fig. 1C and F) 。UCBTERT-21 細胞では約 97%の細胞が 2 コピーの 13 番染色体をもっていることから、染色体構成が安定であることを示した(Fig. 1G)。UE6E7T-3 と UBE6T-6 細胞の染色体数は 43-45 本であり、UE6E7T-3 の 76%が、UBE6T-6 の 86%が 13 番染色体を一本だけ持っていた(Fig. 1I and J)。同様に、UCB408E6E7TERT-33 細胞は 70%が 3 本の 13 番染色体を持った near-tetraploid であった(Fig. 1H)。

さらに FISH 法により観察された染色体 13 番の欠失が CGH アレイ法によって確かめられた。長期培養の初期ステージ（青色スポット）と後期ステージ（赤色スポット）のデータを Fig. 2 に示した。UCBTERT-21 細胞では培養の初期および後期において共に変化が認められなかった(Fig. 2A)。 UBE6T-6 と UCB408E6E7TERT-33 細胞の培養初期に 13 番染色体が欠失していたことを FISH 法で認めたが、CGH 法からはさらに 4 番、9 番、16 番染色体も欠失していることを認めた(Fig. 2B and D)。UE6E7T-3 については、PDL 78 から PDL 101 の間で 16 番染色体の欠失も観られたが、特に特徴的なことはこれら 3 種類の細胞株に共通して 13 番染色体の欠失が観られたことである。これらのデータは FISH 分析の結果と一致した。またより詳細な解析が可能なアジレント社製アレイ CGH 解析においても同様の結果が認められ、再現性の有る非常に貴重なデータが得られた。

### C.2　不死化間葉系幹細胞の分化能

間葉系幹細胞は骨芽細胞、軟骨細胞や脂肪細胞に分化することができるし、ときには神経様細胞に分化したりすることが可能であり、すなわち多分化能を持っていると報告されている。今回用いた 4 種類の細胞株を適切な誘導培地で 2 ～ 4 週間培養した。特に、UE6E7T-3 細胞は他の細胞よりも脂肪細胞へ

分化する能力が強い(Fig. 3Ab)。骨芽細胞への分化能の一番強いのは UCB408E6E7 TERT-33 細胞であった(Fig. 3Ad)。UBE6T-6 細胞は形態学的、マーカー遺伝子発現において神経様細胞への分化を示した。誘導培地で 28 日間培養後、III-β-tubulin 抗体、または neurofilament NF-200 抗体による蛍光染色すると神経突起様構造が観察された(Fig. 3Af and Ah)。一方、誘導培地で処理する前の UBE6T-6 細胞ではこのような構造は見られなかった(Fig. 3Ae and Ag)。これらの結果を Fig. 3B にまとめて記載した。

### C.3　hMSC の詳細解析

Cambrex 社より購入したヒト間葉系幹細胞を継代培養により長期培養維持した後、長期培養の前後での染色体解析を実施した。また、その際用いる培地による影響を検討するため、MSCBM (間葉系幹細胞培養用培地) と DMEM の２種類の培地で培養を行いその比較を行った。その結果どちらの培地の場合においても、増殖速度に違いは見られたものの (DMEM で培養すると増殖速度が落ちる) 、mFISH の染色体写真には異常が観察されなかった。

## D. 考察

本研究では、遺伝子を導入したヒト臍帯血、骨髄間葉系幹細胞が in vitro 培養で遺伝子型、表現型にどのような変化を示すかを解析し明らかにした。本研究で使用した４細胞株（ UCBTERT-21, UCB408E6E7TERT-33, UE6E7T-3, UBE6T- 6）のうち、hTERT のみで不死化した細胞株 UCBTERT-21 は細胞周期の制御に深く関わる p53/p21 や pRB/$p16^{INKA}$ 経路の変異なしに寿命を相当のばすことができた。しかしながら、同じ遺伝子 hTERT で不死化したヒトケラチノサイトは延命したが、pRB/$p16^{INKA}$ 経路の不活性化や p16 発現の downregulation が見られることが報告されている。これらのことから細胞のタイプによって不死化にはテロメア以外のバリヤーが存在する可能性が示された。

一方、ヒトパピローマウイルス E6/E7 遺伝子や Bmi-1 がん遺伝子と h TERT を用いて不死化した細胞株 UCB408E6E7TERT-33（臍帯血由来）、UE6E7T-3 や UBE6T-6（骨髄由来）は不死化したものの長期培養を続けると、染色体数に大きな変化を示した。UCB408E6E7TERT-33 細胞は培養初期には diploid (2n)であるが、培養期間が長くなるにつれて、tetraploid (4n)と tetraploid より少し少ない異数体 aneuploid(４n-１～5)になる。同じようなパターンが UE6E7T-3 や UBE6T-6 でも観察された。最近、ヒト N/TERT-1 ケラチノサイトや HeLa 細胞の in vitro 実験で、異数体 aneuploid が形成される前に tetraploid の形成があり、それは分裂期に２つの娘細胞の不分離によるという nondisjunction 説が報告されたが、古くからヒトがん細胞でも高頻度で tetraploid が観察されている。Aneuploid の UCB408E6E7TERT-33 細胞の染色体構成を CGH 解析や 13 番染色体と 17 番染色体プローブを用いた pFISH で調べた結果は見事に細胞あたり 17 番染色体が４本、13 番染色体が３本であることが明らかになった。さらに驚いたことは、17 番染色体は正常であるのに 13 番染色体の１本の欠失が diploid UE6E7T-3 や UBE6T-6 両細胞の 70～80%で観察されたことである。これらの３細胞株に共通してみられるこの現象から、この培養条件でこれらの細胞株が増殖していくためには 13 番染色体の欠失が重要であったことが示されている。特定染色体の欠失によるカリオタイプ変異はヒト ES 細胞でも報告されている。これまで染色体異数体は tetraploid から形成されるという主張が強かったが、本研究から tetraploid を経由せず、diploid からでも形成されるという異数体形成機構の新たな事実が示された。

tetraploid 形成なしに起こる異数体形成機構は不明であるが、中心体が重要な役割をしている可能性が高い。正常細胞や UCBTERT-21 細胞では、中心体が細胞あたり１～2 個しかないものが、この３細胞株では３～10 個も存在するものが細胞の 12～35%にもおよび、この問題は次の機会に明らかにしたいと考えている。

細胞治療で最も重要な問題は移植する細胞の質的要素である。間葉系幹細胞が今日再生医療面で大きな期待を受けているのは、他の組織幹細胞ではみられないいろいろな他組織への分化能を持っているからである。この研究で用いた細胞株は臍帯血と骨髄由来の幹細胞であるが、in vitro の限られた条件下でも骨細胞、脂肪細胞、神経細胞への分化能を保持していた。遺伝子を導入してもその分化能を維持していた。さらに市販されてい

るヒト間葉系幹細胞に関しても、長期培養における染色体異常解析を行ったが、培地の違いによりその増殖速度に影響を与えることは明らかになったが、長期培養による染色体異常は観察されなかった。今後アレイ CGH による染色体のより詳細な解析を試み、より微細な染色体変化を、より簡便に解析する評価系開発を目指す。

## E. 結論

hTERT を導入した臍帯血由来間葉系幹細胞は 133PDL という長期培養でもカリオタイプに変化は見られず、分化能も保持していた。この結果は再生医療に必要とする細胞の in vitro 増幅の一歩前進になる。一方、がん遺伝子導入細胞株は 13 番染色体 1 本の特異的欠失を伴うが、間葉系細胞本来の分可能は保持していた。これらのことから、これらの細胞株はがん細胞のような染色体不安定性の解析や脂肪細胞、骨細胞を含めた多組織への分可能の研究には最適の材料である。

今後、細胞治療がますます盛んになるが、ES 細胞だけではなく、組織幹細胞でも移植に必要な細胞量の確保には in vitro 増幅が不可欠である。そのためには他細胞のコンタミネーションやマイコプラズマ汚染、ウイルス汚染チェックと同じように、移植された組織の悪性変異を防ぐためにもカリオタイプの検査は品質管理の重要な項目に加えなければならないことをこの研究は警告している。

## F. 健康危険情報

該当事項なし

## G. 研究発表

### 1. 論文発表

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2) Arihiro Kohara; High-Resolution genomic analysis of immortalized human cells and human tumor cells using array-based comparative genomic hybridization; The 8th International Symposium on Chromosomal Aberrations; 2007 年 10 月 (兵庫)

3) Arihiro Kohara; High-resolution genomic analysis of immortalized human cells and human tumor cells using array-based comparative genomic hybridization; 47th ASCB Annual Meeting; 2007 年 12 月 (Washington, DC, USA)

## H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

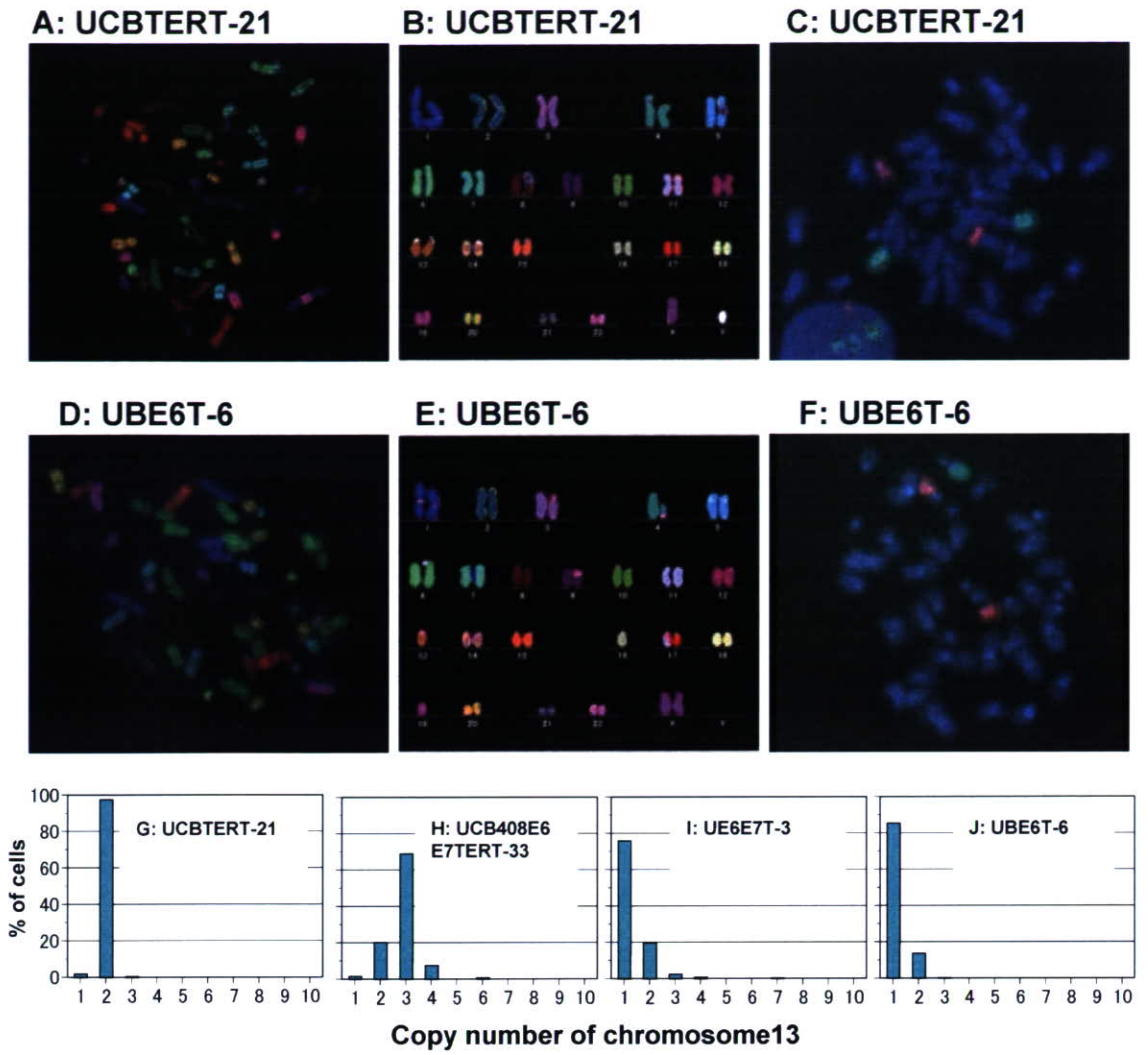



Fig. 1　遺伝子導入したヒト間葉系幹細胞株の FISH 分析
UCBTERT-21 (A, B, C) とUBE6T-6 (D, E, F)の染色体の mFISH (A, D)、カリオタイプ (B, E) および染色体 13 番 (green) および 17 番 (red)(C, F) の pFISH 画像を示した。G - J、4 種類の細胞株での細胞あたり 13 番染色体のコピー数。FISH シグナルは 120～200 の展開した染色体と間期核中のスポットを測定した。 UCBTERT-21 細胞は 2 コピーの 13 番および 17 番染色体を持っている。他の細胞では 13 番染色体の 1 コピーが欠失している。

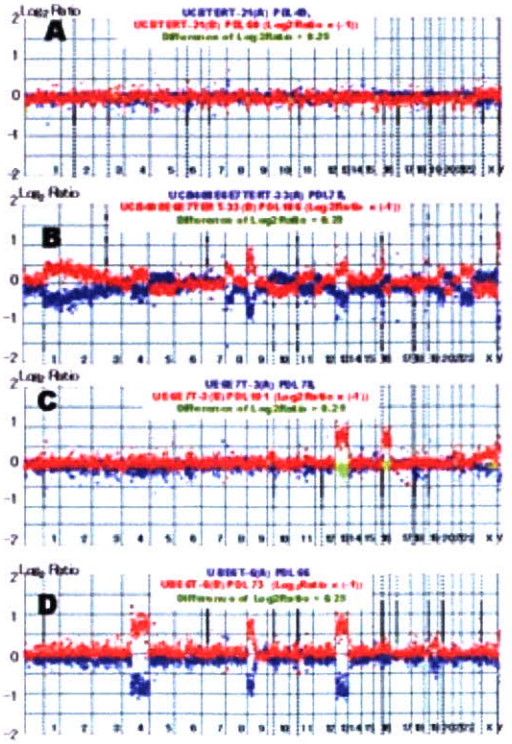
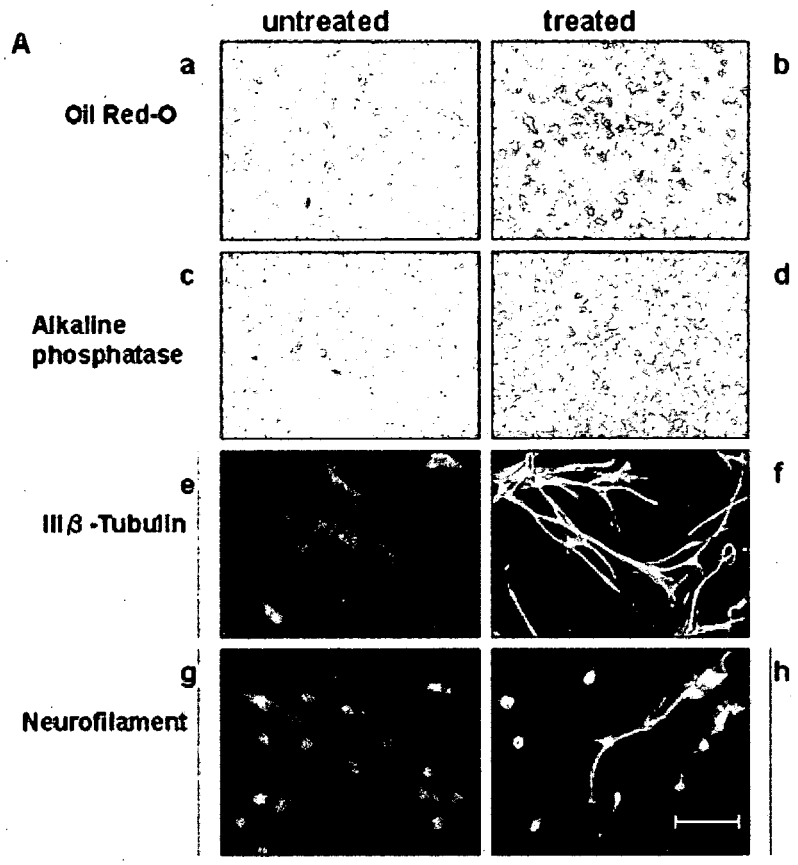



Fig. 2　培養初期と後期における 4 種類の遺伝子導入ヒト細胞株の CGH アレイプロファイル
X 軸は 22 組の常染色体、X と Y 染色体 chromosomes を Y 軸は [cy3 (hMSCs) / cy5 (normal cell)] の蛍光強度比を log2 で示した。0 より上 (red spots) または 0 より下 (blue spots) は その染色体領域の欠失を示した。青スポットは培養の初期のステージ、赤スポットは後期ステージを示し、それぞれをオーバーレイした。青と赤の差のあるスポットを緑で示した。UE6E7T-3 細胞については PDL 78 と 101 の間で 13 番と16 番染色体の各 1 本づつ欠失が見られた。




B

| | UCBTERT-21 | UCB408E6E7TERT-33 | UE6E7T-3 | UBE6T-6 |
|---|---|---|---|---|
| Oil Red-O | + | ++ | +++ | + |
| Alkarine phosphatase | + | +++ | + | + |
| III β-Tubulin | − | + | +/- | +++ |
| Neurofilament | − | ++ | − | ++ |

Fig. 3　遺伝子導入したヒト間葉系幹細胞株の脂肪細胞、骨芽細胞、神経様細胞への分化
A:　分化した脂肪細胞は Oil Red-O 染色による染色顆粒の蓄積が見られる (Aa and Ab, UE6E7T-3 細胞)。 骨芽細胞は高い alkaline phosphatase 活性 (赤) (Ac and Ad, UCB408E6E7TERT-33細胞)を示し、神経細胞は Ⅲβ-tubulin と neurofilament の特異的タンパク質の発現 (免疫抗体染色) および細胞の形態変化により示された(Ae - Ah, UBE6T-6細胞)。
B:　4 種類の細胞株について胞分化能の違いの比較を示した。
－；未処理細胞と同等、　＋；弱い反応性、　＋＋＋； 高い反応性　(Bar は20 μm)

研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
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# Efficient adenovirus vector-mediated PPAR gamma gene transfer into mouse embryoid bodies promotes adipocyte differentiation

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## Abstract

**Background** Establishment of a transient gene delivery system, such as adenovirus (Ad) vectors, into embryonic stem (ES) cells and their aggregation form, embryoid bodies (EBs), is essential for its application in regenerative medicine because the transgene should not be integrated in the host genome. In this study, we optimized Ad vector-mediated transduction into EBs, and examined whether Ad vector-mediated transduction of adipogenesis-related gene into EBs could promote the adipocyte differentiation.

**Methods** We prepared $\beta$-galactosidase-expressing Ad vectors under the control of four different promoters (cytomegalovirus (CMV), rouse sarcoma virus, human elongation factor-1$\alpha$, and CMV enhancer/$\beta$-actin promoter (CA)) to estimate the transduction efficiency. Adipocyte differentiation efficiency by transduction of the PPAR gamma or C/EBP alpha gene into EBs was examined.

**Results** Of the four promoters tested, the CA promoter exhibited the highest transduction efficiency in the EBs. However, Ad vector-mediated transduction was observed only in the periphery of the EBs. When repeated transduction by Ad vector was performed, gene expression was observed even in the interior of EBs as well. When EB-derived single cells were transduced by an Ad vector containing the CA promoter, more than 90% of the cells were transduced. Furthermore, Ad vector-mediated PPAR gamma gene transduction into EBs led to more efficient differentiation into adipocytes than could untransduced EBs, examined in terms of lipogenic enzyme activities and accumulation of the lipid droplets.

**Conclusions** Ad vector-mediated transduction into EBs could be a valuable tool for molecular switching of cell differentiation and could be applied to regenerative medicine. Copyright © 2008 John Wiley & Sons, Ltd.

**Keywords** adenovirus vector; embryonic stem cells; embryoid bodies; regenerative medicine

## Introduction

Embryonic stem (ES) cells are derived from mammalian blastocysts and maintain pluripotency, an ability to differentiate into all types of somatic and germ cells. Another important property of ES cells is their robust and infinite growth, equivalent to tumor cells in spite of their normal karyotype. Mouse ES (mES) cells were isolated from

mouse blastocysts in 1981 [1] and have been extensively used to generate knockout mice. Human ES cells were established in 1998 [2] and are considered promising sources for cell transplantation therapy.

ES cells differentiate spontaneously *in vitro* in a random fashion into all three germ layers. Therefore, establishment of the differentiation protocols from ES cells into pure target cells is expected to be applicable to regenerative medicine. Among many methods for inducing cellular differentiation from ES cells, genetic manipulation is one of the most powerful techniques to control cellular differentiation. Long-term constitutive gene expression systems such as electroporation methods and a retrovirus vector system by which antibiotic-resistant stable cells are established have been developed and utilized so far to differentiate ES cells into committed cells and to analyze gene function [3–7]. However, such expression systems may be problematic especially in therapeutic application because the transgene is randomly integrated into the host cell genome and this leads to a risk of mutagenesis [8]. Therefore, instead of a long-term constitutive gene expression system, establishment of a transient expression system is required for differentiation from ES cells into functional cells.

Among the various types of gene delivery vectors, adenovirus (Ad) vectors based on human Ad type 5 (hAd5) have been widely used for gene delivery, since they can be amplified at high titers, have the ability to package relatively large-sized foreign DNA, and achieve high transduction efficiency [9,10]. Furthermore, in contrast to stable gene expression, only little genomic DNA of the Ad vector is integrated into the host cell DNA, and its expression is transient. These features of the Ad vector are thought to be advantageous for cellular differentiation since transgene expression is not often needed for the cells after differentiation. From such a viewpoint, we previously reported efficient transduction into mES cells using an Ad vector [11].

When ES cells differentiate into functional cells, they are suspended in the medium on non-adherent culture plates or in hanging drops. The suspended ES cells spontaneously aggregate to form spheres, called embryoid bodies (EBs), which consist of semi-organized tissue including contractile cardiac myocytes and hemoglobin-containing blood islands [12]. Although the procedures to regulate ES cell differentiation are often carried out through EBs [5,7], no study has been performed investigating the transduction efficiency for EBs.

In the present study, we optimized transduction efficiency through comparison of the promoter activities in EBs by using $\beta$-galactosidase (LacZ)-expressing Ad vectors. Furthermore, to test whether the differentiation efficiency of functional cells from EBs could be improved by using an Ad vector-mediated gene transfer, we introduced a peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) gene, which has been shown to be indispensable for adipogenesis [13,14], or a CCAAT/enhancer binding protein alpha (C/EBP$\alpha$) gene, which has also been shown to be a key transcription factor for adipogenesis [15,16], into EBs.

# Materials and methods

## Plasmid construction and generation of Ad vectors

Ad vectors were constructed using an improved *in vitro* ligation method [17,18]. The murine PPAR$\gamma$1 gene, which is derived from pHMCMV6-PPAR$\gamma$1 (a kind gift from Dr. K. Katayama, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) [19], was digested with *Xba*I and *Not*I, and inserted between the *Xba*I and *Not*I sites of pHMCA5 [11], resulting in pHMCA5-PPAR$\gamma$1. pHMCA5-PPAR$\gamma$2 was constructed by insertion of the oligonucleotides 5′-catgggtgaaactctgggagattctcctgtagacccagagcatggtgcctt cgctgatgcactgcctatgagcacttcacaagaaattaccatggta-3′ and 5′-taccatggtaatttcttgtgaagtgctcataggcagtgcatcagcgaagg caccatgctctgggtctacaggagaatctcccagagtttcacc-3′ (underlined sequences indicate the mutated *Hinc*II site with silent mutation to prevent cleaving) into the *Nco*I and *Hinc*II sites of pHMCA5-PPAR$\gamma$1, because murine PPAR$\gamma$2 cDNA encodes an additional thirty amino acids N-terminal to the first ATG of murine PPAR$\gamma$1 [20]. Murine C/EBP$\alpha$ cDNA, which is derived from pEF-C/EBP$\alpha$ (a kind gift from Dr. M. Takiguchi, Chiba University, Chiba, Japan) [21], was digested with *Bst*XI, blunted by a Klenow fragment of DNA polymerase, and cloned into the *Pme*I site of pHMCA5, resulting in pHMCA5-C/EBP$\alpha$. pHMCA5-PPAR$\gamma$1, pHMCA-PPAR$\gamma$2, or pHMCA5-C/EBP$\alpha$ was then digested with I-*Ceu*I/PI-*Sce*I and inserted into I-*Ceu*I/PI-*Sce*I-digested pAdHM4 [17], resulting in pAdHM4-CA-PPAR$\gamma$1, pAdHM4-CA-PPAR$\gamma$2, or pAdHM4-CA-C/EBP$\alpha$, respectively.

To generate the virus, Ad vector plasmids were digested with *Pac*I and were then transfected into 293 cells plated in 60 mm dishes with SuperFect (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The virus was purified by $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −80 °C. The rous sarcoma virus (RSV) promoter, the cytomegalovirus (CMV) promoter, the CMV enhancer/$\beta$-actin promoter (CA) promoter, or the human elongation factor-1$\alpha$ (EF-1$\alpha$) promoter-driven LacZ-expressing Ad vector, Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ, respectively, and CA promoter-driven green fluorescent protein (GFP)-expressing Ad vector, Ad-CA-GFP, were constructed previously [11,22]. Determination of virus particles (VP) and biological titer were determined using a spectrophotometric method [23] and by means of an Adeno-X rapid titer kit (Clontech, Palo Alto, CA, USA), respectively. The ratio of the biological-to-particle titer was 1 : 14 for Ad-CA-LacZ, which was re-amplified in 293 cells to use in this study, 1 : 8 for Ad-CA-PPAR$\gamma$1, 1 : 8 for Ad-CA-PPAR$\gamma$2, and 1 : 9 for Ad-CA-C/EBP$\alpha$.



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## Cell culture and EB formation

Mouse E14 ES cells were cultured on mytomycin C-treated mouse embryonic fibroblasts (MEFs) or on a gelatin-coated plate in a leukemia inhibitory factor-containing ES cell culture medium as described previously [11]. To induce formation of EBs, mES cells on MEFs were trypsinized, and MEF layers were separated from mES cells by culturing at 37 °C for 45 min. Nonadherent cells, which contain undifferentiated ES cells, were resuspended in differentiation medium (Dulbecco's modified Eagle's medium (WAKO, Osaka, Japan) containing 15% fetal calf serum (Specialty Media, Inc., Phillipsburg, NJ, USA), 0.1 mM 2-mercaptoethanol (Nacalai tesque, Kyoto, Japan), 1× non-essential amino acid (Specialty Media, Inc.), 1× nucleosides (Specialty Media, Inc.), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, uSA), and penicillin/streptomycin (Invitrogen)) at a concentration of $1 \times 10^5$ cells/ml, and $3 \times 10^3$ cells were cultured on the inner side of 100 mm Petri dish lids (hanging drop method) and incubated at 37 °C for 2 or 5 days.

Five-day-cultured EBs (5d-EBs) were harvested, washed with phosphate-buffered saline (PBS), and incubated in 1×trypsin/EDTA (Invitrogen) at 37 °C for 5 min. EBs were dissociated in differentiation medium by repeated pipetting and passing through a 20-gauge needle. The single cell suspension was kept on ice for further analysis.

## LacZ assay

5d-EBs were transduced with the indicated doses of conventional Ad vectors (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ or Ad-EF-LacZ) at 37 °C. Two days later, X-gal staining and $\beta$-gal assays were performed as described previously [11]. The EB-derived single cell suspension was transduced with the indicated doses of Ad-CA-LacZ at 37 °C for 1.5 h before plating. The cells were then washed with PBS and plated on gelatin-coated dishes. On the following day, X-gal staining was carried out as described above.

## GFP expression analysis

EBs were transduced with the Ad-CA-GFP at 10 000 VP/cell. At 1.5 h after incubation, the cells were washed to remove the Ad vectors and were transferred into fresh medium. The EBs were transduced with 10 000 VP/cell of Ad-CA-GFP three times on days 0, 2, and 5 (hereinafter referred to as triple transduction), as follows: 0d-EBs (ES cells suspension) were transduced with Ad vector in hanging drop for 2 days, and 2d-EBs and 5d-EBs were transduced with Ad vector for 1.5 h. On day 7, GFP fluorescence in the EBs was visualized via confocal microscopy (Leica TCS SP2 AOBS; Leica Microsystems, Tokyo, Japan). The EBs were then trypsinized and analyzed for GFP expression by flow cytometry on a FACSCalibur flow cytometer using CellQuestPro software (Becton Dickinson, Tokyo, Japan)

## Adipocyte differentiation with Ad vector

Two days after culture with hanging drop, the EBs were transferred into a Petri dish and maintained for 3 days in suspension culture in differentiation medium containing 100 nM all-*trans*-retinoic acid (RA, WAKO), and then cultured for 2 more days in differentiation medium without RA [24]. The cells were transduced with 10 000 VP/cell of Ad vectors (Ad-CA-LacZ, Ad-CA-PPAR$\gamma$1, Ad-CA-PPAR$\gamma$2 or Ad-CA-C/EBP$\alpha$) at days 0, 2, and 5 as described above and plated on a gelatin-coated dish on day 7. Cells were cultivated in differentiation medium with or without adipogenic supplements (0.1 M 3-isobutyl-L-methylxanthine (Sigma, St. Louis, MO, USA), 100 nM insulin (Sigma), 0.1 μM dexametasone (WAKO), and 2 nM triiodothyronine (Sigma)) and the medium was changed every 2 or 3 days.

Differentiation of EBs into adipocytes was estimated by Oil-red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity on days 12 and 24 after plating. Oil-red O staining and a GPDH assay were performed using a lipid assay kit and GPDH assay kit, respectively (Cellgarage, Hokkaido, Japan). For the analysis of lipid accumulation, stained lipid was extracted with 100% isopropanol for 5 min and the optical density of the solution was measured at 540 nm. For the GPDH assay, protein content was determined using an assay kit (Bio-Rad Laboratories, Hercules, CA, USA) employing bovine serum albumin as a standard, and GPDH activities were then normalized to protein content.

## Western blotting

ES cells, 2d-EBs, and 5d-EBs were lysed in lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease inhibitor cocktail (Sigma). Lysates (20 μg) were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking with 3% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 2 h, the membrane was incubated with goat anti-CXADR (coxsackievirus and adenovirus receptor, hereinafter referred to as CAR) antibody (R&D Systems, Minneapolis, MN, USA, diluted 1:1000) or mouse anti-Oct-3/4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, diluted 1:200) at 4 °C overnight, followed by horseradish peroxidase conjugated anti-goat IgG (Chemicon, Temecula, CA, USA) or anti-mouse IgG (Cell Signaling Technology, Beverly, MA, USA), respectively, at room temperature for 1 h. The band





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was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ, USA) and the signals were read using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). For the detection of internal control, a monoclonal anti-$\beta$-actin antibody (Sigma, diluted 1 : 5000) and a horseradish peroxidase conjugated anti-mouse IgG were used.

## Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated as described previously [11]. DNaseI-treated samples were reverse-transcribed using SuperScript II (Invitrogen), and PCR was then performed using KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The PCR conditions were 94 °C for 2 min, followed by appropriate cycles of 94 °C for 15 s, 55 °C for 30 s with 68 °C for 30 s and a final extension of 68 °C for 1 min, except for the addition of 5% dimethyl sulfoxide in the case of C/EBP$\alpha$ and leptin cDNA amplification. PCR products were visualized by ethidium bromide staining after being separated on 2% agarose gel. The sequences and references of primers were as follows: PPAR$\gamma$(F), 5′-CCCTGGCAAAGCATTTGTAT-3′; PPAR$\gamma$(R), 5′-AATCCTTGGCCCTCTGAGAT-3′; C/EBP$\alpha$(F), 5′-CGCTGGTGATCAAACAAGAG-3′; C/EBP$\alpha$(R), 5′-GTCACTGGTCAACTCCAGCA-3′; aP2(F), 5′-TGGAAGCTTGTCTCCAGTGA-3′; aP2(R), 5′-ACACATT CCACCACCAGCTT-3′; adiponectin(F), 5′-GTTGCAAG CTCTCCTGTTCC-3′; adiponectin(R), 5′-GCTTCTCCAGG CTCTCCTTT-3′; leptin(F), 5′-TGACACCAAAACCCTCA TCA-3′; leptin(R), 5′-CTCAAAGCCACCACCTCTGT-3′, CAR, Oct-3/4, Nanog, Brachyury T, GAPDH and LacZ were described previously [11,25].

# Results

## Transgene expression in EBs by Ad vectors

Initially, we characterized the EBs used in this study by examining the expression of cellular marker genes. Consistent with previous reports, the expression of Nanog and Oct-3/4, both of which are transcription factors involved in the maintenance of pluripotency in mES cells, were down-regulated following EB formation, whereas the expression of brachyury T, the early pan-mesodermal marker, was detectable in EBs (Figures 1A and 1B) [26,27]. It is known that expression of CAR, a primary receptor of Ad, is essential for Ad vector-mediated gene transduction [10]. To confirm whether EBs could be efficiently transduced with Ad vectors, we assessed the expression of CAR in EBs (Figures 1A and 1B). We found that expression levels of CAR in both 2d-EBs and 5d-EBs (EBs cultured for 2 or 5 days, respectively) were similar to those of mES cells, suggesting that exogenous genes could be introduced into EBs by using a conventional Ad vector.

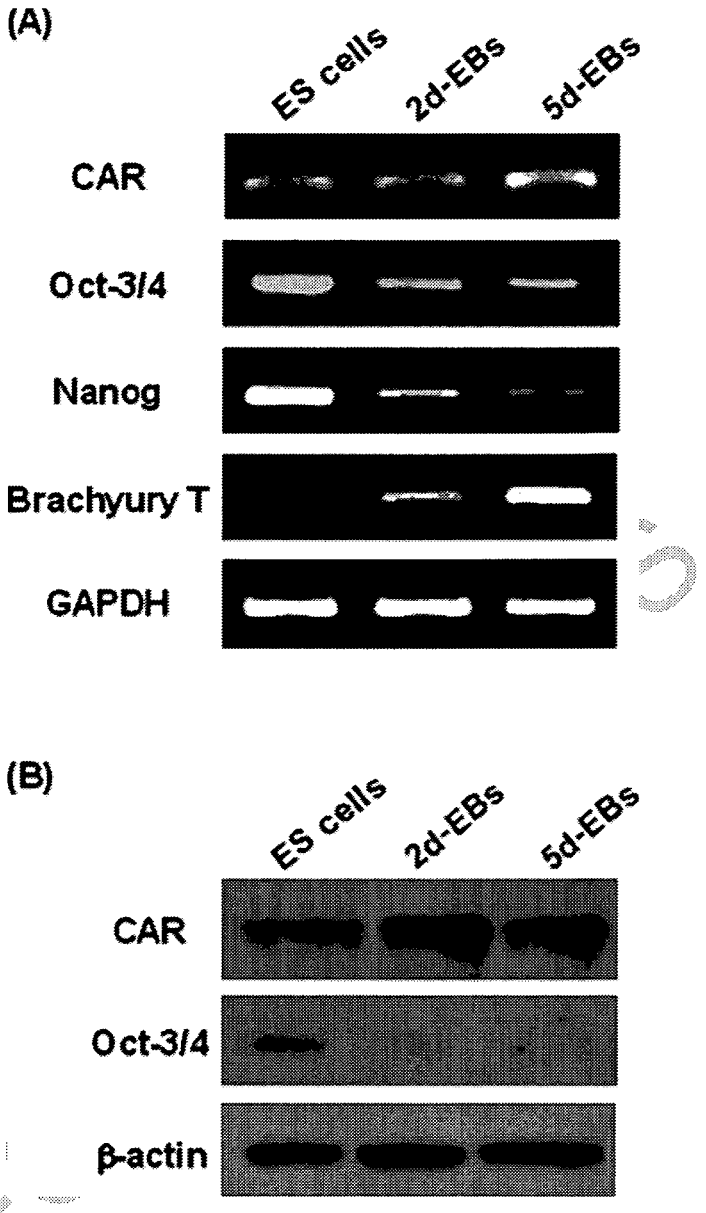


**Figure 1. RT-PCR and Western blot analysis of ES cells and EBs. Total RNA or whole cell lysates were isolated from ES cells (lane 1), 2d-EBs (lane 2), or 5d-EBs (lane 3). RT-PCR (A) and Western blotting (B) were carried out as described in Materials and Methods. Abbreviations: ES cells, embryonic stem cells; EBs, embryoid bodies; 2d-EBs, two-day-cultured EBs; 5d-EBs, five-day-cultured EBs; CAR, coxsackievirus and adenovirus receptor**

We next prepared LacZ-expressing Ad vectors under the control of four different promoters, the RSV promoter, the CMV promoter, the CA promoter, or the EF-1$\alpha$ promoter (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ, respectively) to optimize the efficiency of transgene expression in EBs. 5d-EBs were transduced with each Ad vector (3000 virus particles (VP)/cell) and LacZ expression in the cells was measured. As shown in Figures 2A and 2B, Ad-CA-LacZ-transduced EBs showed

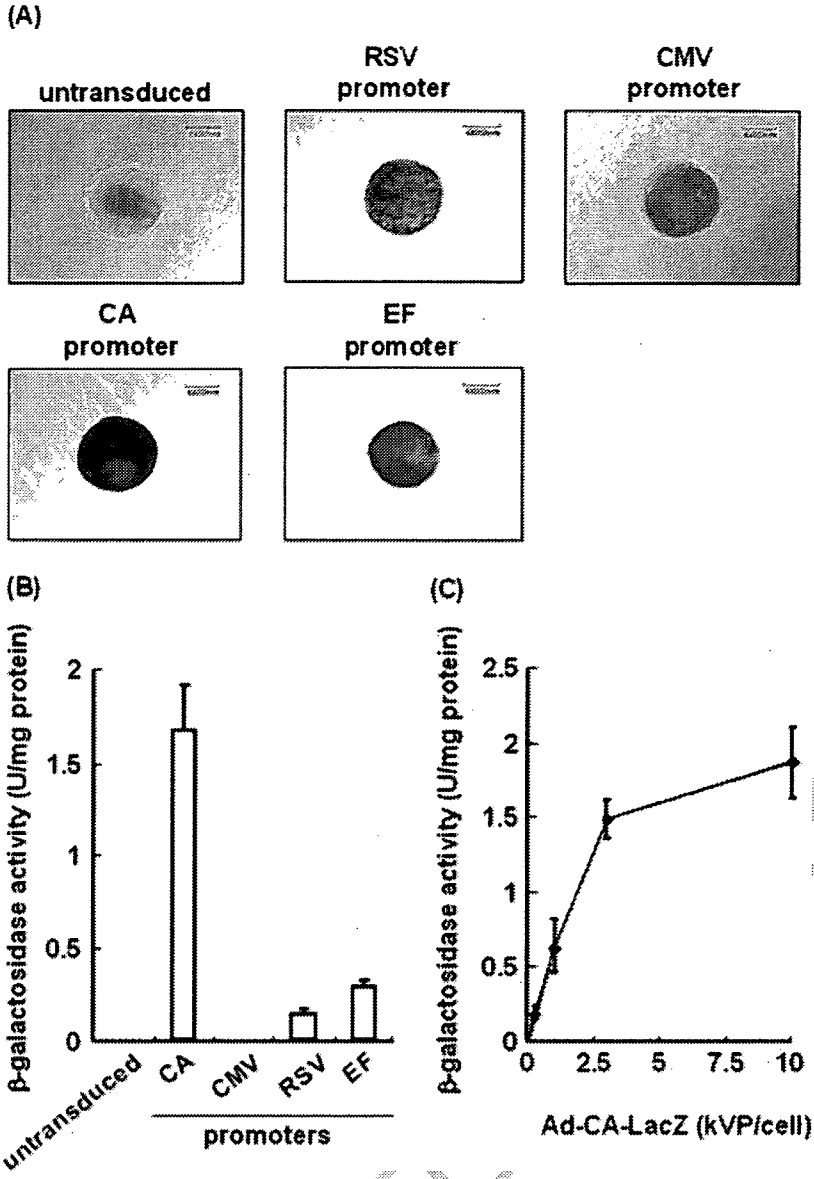


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**Figure 2. Ad vector-mediated transduction efficiency in EBs as determined using various types of promoters. 5d-EBs were transduced with Ad vectors at 3000 VP/cell for 2 days. After 48 h, X-gal staining (A) and β-galactosidase luminescence assay (B) were performed as described in Materials and Methods. Similar results for X-gal staining were obtained in five independent experiments. (C) 5d-EBs were transduced with 300, 1000, 3000, or 10 000 VP/cell of Ad-CA-LacZ for 2 days. Two days after transduction, LacZ expression in the cells was measured by luminescence assay. The data (B and C) are expressed as mean ± standard deviation (S.D.) (n = 3). Abbreviations: RSV, rous sarcoma virus; CMV, cytomegalovirus; CA, CMV enhancer/β-actin promoter; EF-1α, human elongation factor-1α**

greater LacZ expression than did Ad-RSV-LacZ- or Ad-EF-LacZ-transduced EBs. Although the CMV promoter is in wide use in transduction experiments, Ad-CMV-LacZ-transduced EBs showed little expression of LacZ. These data indicate that the transduction efficiency in EBs is dependent on the promoter and that the CA promoter is the most active in EBs among the four types of promoters examined in this study.

To determine an appropriate dose of Ad-CA-LacZ for the transduction efficiency in EBs, 5d-EBs were transduced with a different dose of Ad-CA-LacZ for 2 days, and then LacZ production in the cells was quantified by means of a luminescence assay. The expression of LacZ in the EBs increased depending on the dose of Ad vectors and reached a plateau at 3000–10 000 VP/cell (Figure 2C). To obtain high transgene expression, the concentration of Ad vector with 10 000 VP/cell was employed for further analysis. Next, we examined whether an increase in the efficiency of LacZ expression could be obtained in EBs by using fiber-modified Ad vectors. We generated Ad-RGD-CA-LacZ and AdK7-CA-LacZ, which contain the Arg-Gly-Asp (RGD) peptide in the HI loop of the fiber knob [28] and seven tandem lysine residues (K7) in the C-terminal of the fiber knob [29], respectively. These Ad vectors transduce cells through αv integrin and heparan sulfates, respectively, even if cells lack CAR expression. 5d-EBs were transduced with 1000, 3000, or 10 000 VP/cell of Ad-CA-LacZ, AdRGD-CA-LacZ, or



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AdK7-CA-LacZ for 2 days and a luminescence assay for the measurement of LacZ expression was performed. The amount of LacZ expression obtained by using fiber-modified Ad vectors was comparable to that obtained by using a conventional Ad vector (data not shown). Thus, these results indicate that the conventional Ad vector containing the CA promoter is the most suitable vector for transduction to EBs.

Next, 5d-EBs were transduced with 10 000 VP/cell of CA promoter-driven GFP-expressing Ad vector, Ad-CA-GFP, to examine whether transgene expression could be observed inside the EBs. Confocal microscopic analysis revealed GFP expression only at the periphery of the EBs (Figure 3A, middle). The percentage of GFP-expressing cells in the EBs was $25.3 \pm 2.3\%$ as determined by flow cytometric analysis (Figure 3B, middle). A similar pattern of transgene expression was observed in the X-gal staining of sliced sections of EBs transduced with Ad-CA-LacZ (data not shown). These results suggest that Ad vectors do not transduce the cells in the interior of EBs because of the physical barrier constituted by their tight connection. Therefore, repeated transduction of Ad vectors was attempted to express the transgene in the EB interior. First, a transgene was introduced into mES cells but not EBs by Ad vector in hanging drop. After transduction into ES cells under the hanging drop, the 2d-EBs and 5d-EBs were transduced with Ad vector again. Thus, the EBs were transduced with Ad vector three times in total (triple transduction: see Materials and Methods). When the EBs were transduced with Ad-CA-GFP by triple transduction, GFP expression was observed even in the interior of the EBs at day 7, although not all the EB cells showed GFP expression (Figure 3A, right). Furthermore, flow cytometric analysis showed that the GFP-positive cells were significantly increased to $39.2 \pm 4.3\%$ ($p < 0.05$ vs. single infection) (Figure 3B, right), although the transduced cells would be diluted due to their cell division. When the number of cells composed of 7d-EBs was measured, there was almost no difference in cell numbers between untransduced EBs and GFP-transduced EBs by triple transduction (data not shown). In addition, 7d-EBs transduced with Ad vector by triple transduction as well as untransduced EBs could differentiate into adipocytes (Figures 4 and 5), showing that Ad vectors have no cytotoxicity to EBs. These results indicate that triple transduction by using Ad vector is effective to express the transgene in the interior of EBs.

As reported previously, hematopoietic differentiation from ES cells via EBs has been usually performed using an EB-derived single cell [7]. To investigate whether the Ad vector was able to be efficiently introduced into EB-derived single cells, EB-derived single cells, which were obtained by typsinization of 5d-EBs, were transduced with Ad-CA-LacZ (Figure 3C). LacZ expression in the EB-derived single cells was dose-dependent, and more than 90% of the cells expressed LacZ at 3000 VP/cell, demonstrating that the EB-derived single cells efficiently expressed LacZ by transduction using Ad vector containing the CA promoter.

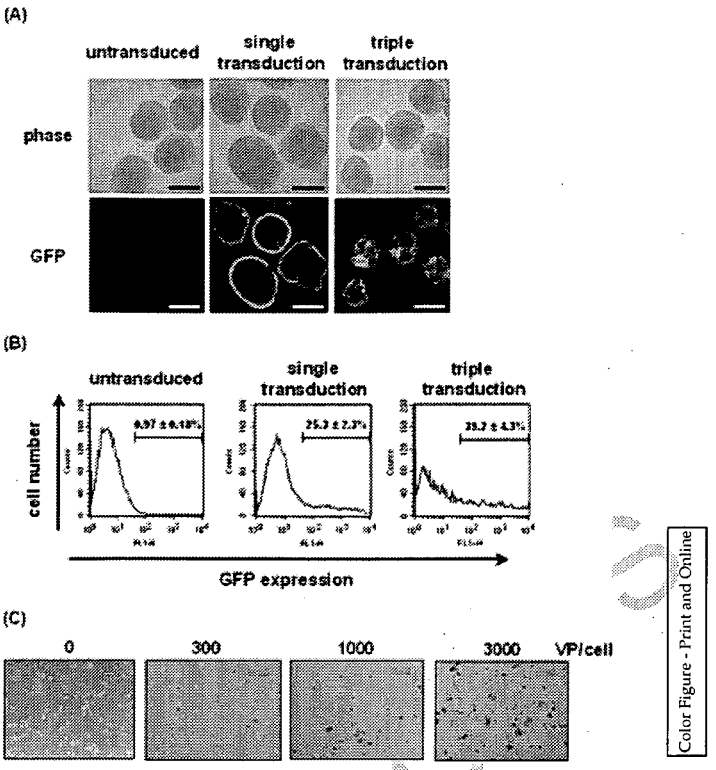


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**Figure 3. Optimization of gene transfer into EBs by Ad vector. EBs were transduced with 10 000 VP/cell of Ad-CA-GFP by single transduction (A, B; middle) or triple transduction (A, B; right). On day 7, (A) confocal microscopic analysis and (B) flow cytometric analysis were performed. The data are expressed as mean ± S.D. ($n = 5$). Untransduced EBs are represented as a negative control (A, B; left). Scale bar indicates 300 μm. (C) Trypsinized 5d-EBs, obtained by trypsin treatment of 5d-EBs, were transduced with Ad-CA-LacZ at doses of 0, 300, 1000, or 3000 VP/cell. On the following day, X-gal staining was performed. Similar results were obtained in three independent experiments. Scale bar indicates 200 μm. Abbreviation: GFP, green fluorescent protein**

## Regulation of cellular differentiation using Ad vector-mediated gene delivery

To confirm that Ad vector-mediated transduction was applicable to basic research or regenerative medicine, we introduced functional genes, which regulate cellular differentiation, into EBs. As a model for cellular differentiation, EBs were differentiated into adipocytes by using Ad vector-mediated transduction of an adipogenesis-related gene. We constructed three Ad vectors, Ad-CA-PPARγ1, Ad-CA-PPARγ2, and Ad-CA-C/EBPα, which expressed murine PPARγ1, PPARγ2, and C/EBPα, respectively. PPARγ and C/EBPα have been shown to play essential roles in adipogenesis [13–16,30]. PPARγ is present in two isoforms, PPARγ1 and PPARγ2, generated by alternative promoter usage [20]. PPARγ2 has an additional thirty N-terminal amino acids relative to PPARγ1. We used both PPARγ1 and PPARγ2 since both could drive a full program of adipogenesis in cultured PPARγ-deficient cells [31]. No study has directly compared the adipogenesis ability, especially adipocyte differentiation from mES cells, of PPARγ1, PPARγ2, and C/EBPα.

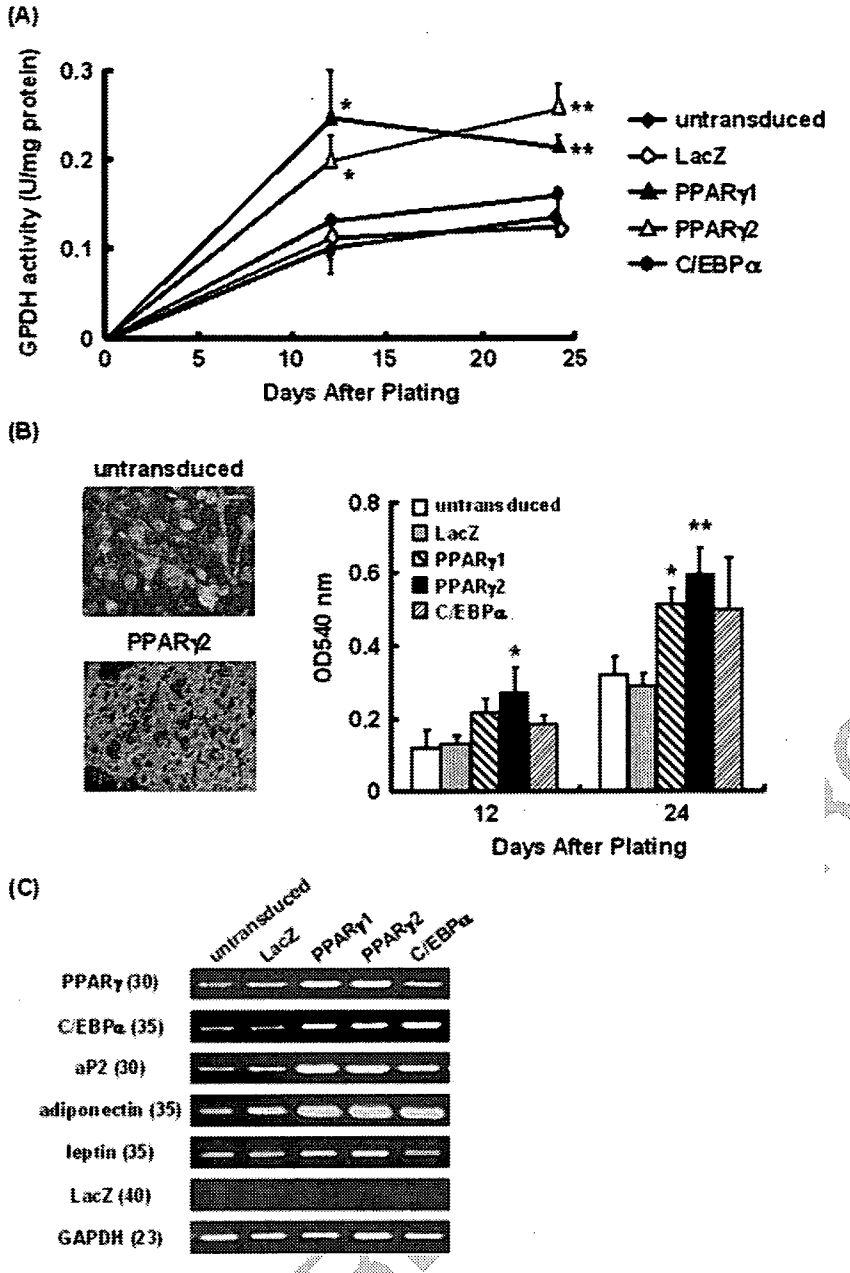


Figure 4. Efficient adipocyte differentiation from EBs by Ad vector-mediated PPARγ gene transfer. EBs were transduced in triplicate with 10 000 VP/cell of Ad-CA-LacZ, -PPARγ1, -PPARγ2, or -C/EBPα. After plating onto a gelatin-coated dish on day 7, EBs were cultured for 24 days with adipogenic supplements. On days 12 and 24 after cultivation GPDH activity in the cell was measured (A). The data are expressed as mean ± S.D. (n = 4). (B) Lipid accumulation was detected by Oil-red O staining at day 24 in the untransduced cells (left, top) or PPARγ2-expressing cells (left, bottom). Scale bar indicates 60 μm. After staining with Oil-red O, stained lipid was extracted and the absorbance at 540 nm was measured (right). The data are expressed as mean ± S.D. (n = 4). (C) The expression of PPARγ, C/EBPα, aP2, adiponectin, leptin, and GAPDH was measured by semi-quantitative RT-PCR. The primer for PPARγ amplified both PPARγ1 and PPARγ2. Cycle number is indicated in parentheses. $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively, as compared with untransduced EBs. Abbreviations: GPDH, glycerol-3-phosphate dehydrogenase; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha

The procedure for adipocyte differentiation from mES cells was carried out as reported by Dani *et al.* [24] except for the step of Ad vector-mediated gene transfer. First, 7d-EB-derived single cells, which were prepared by trypsinization of 7d-EBs, were transduced with Ad-CA-LacZ, -PPARγ1, -PPARγ2, or -C/EBPα because Ad vectors could efficiently introduce a transgene into EB-derived single cells, as shown in Figure 3C. Then, to estimate adipocyte differentiation, GPDH activities in the cells were measured after 24 days cultivation with adipogenic supplements. Although PPARγ-transduced cells exhibited a high level of GPDH activity compared to untransduced cells or LacZ-transduced cells, it was approximately 50-fold lower than that of the untransduced sphere form of EBs, which had not been obtained by trypsin treatment, but was cultured in differentiation medium with adipogenic supplements (data not shown). It is possible that cell-cell interaction in the sphere form of EBs





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