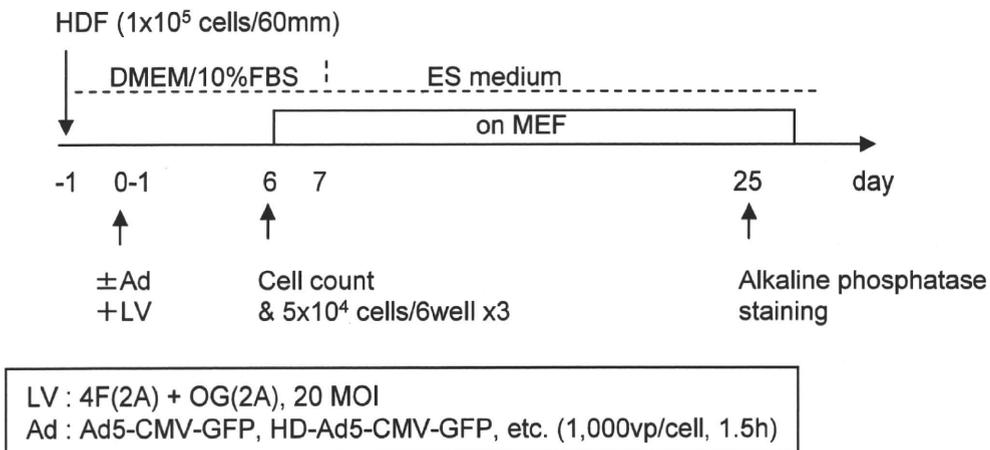
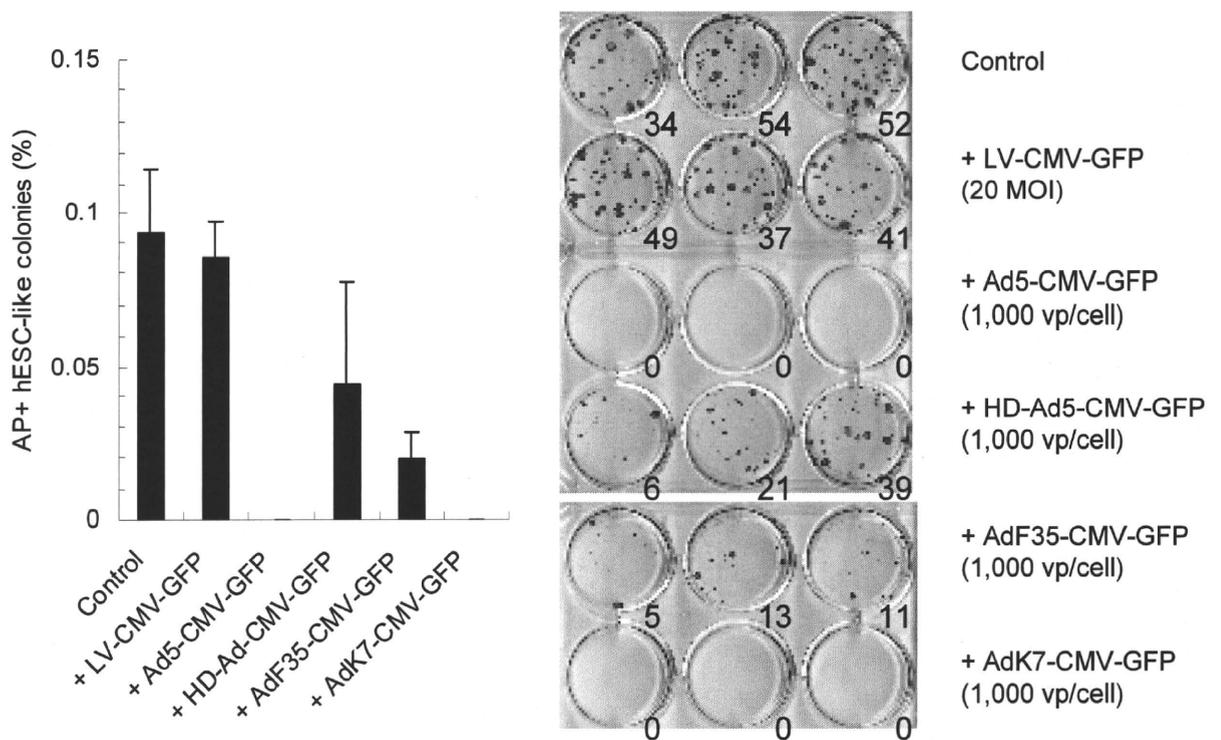


(A)



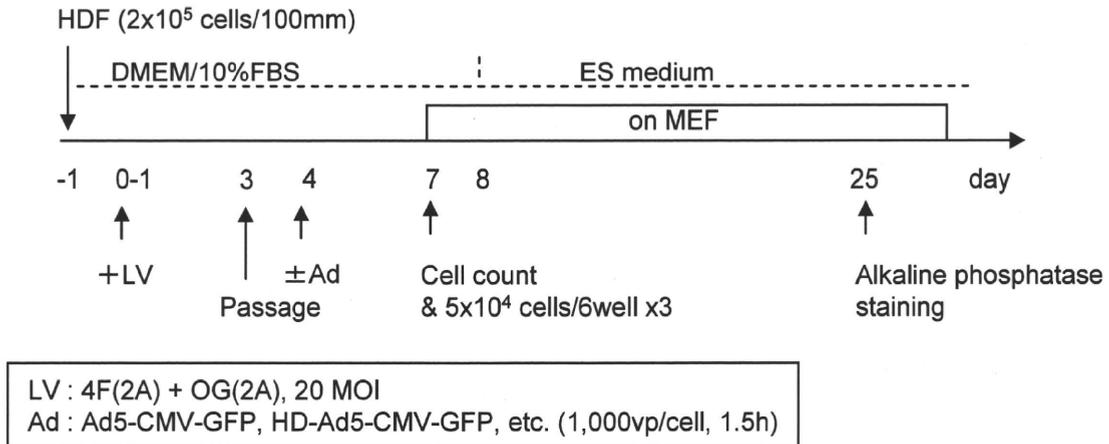
(B)



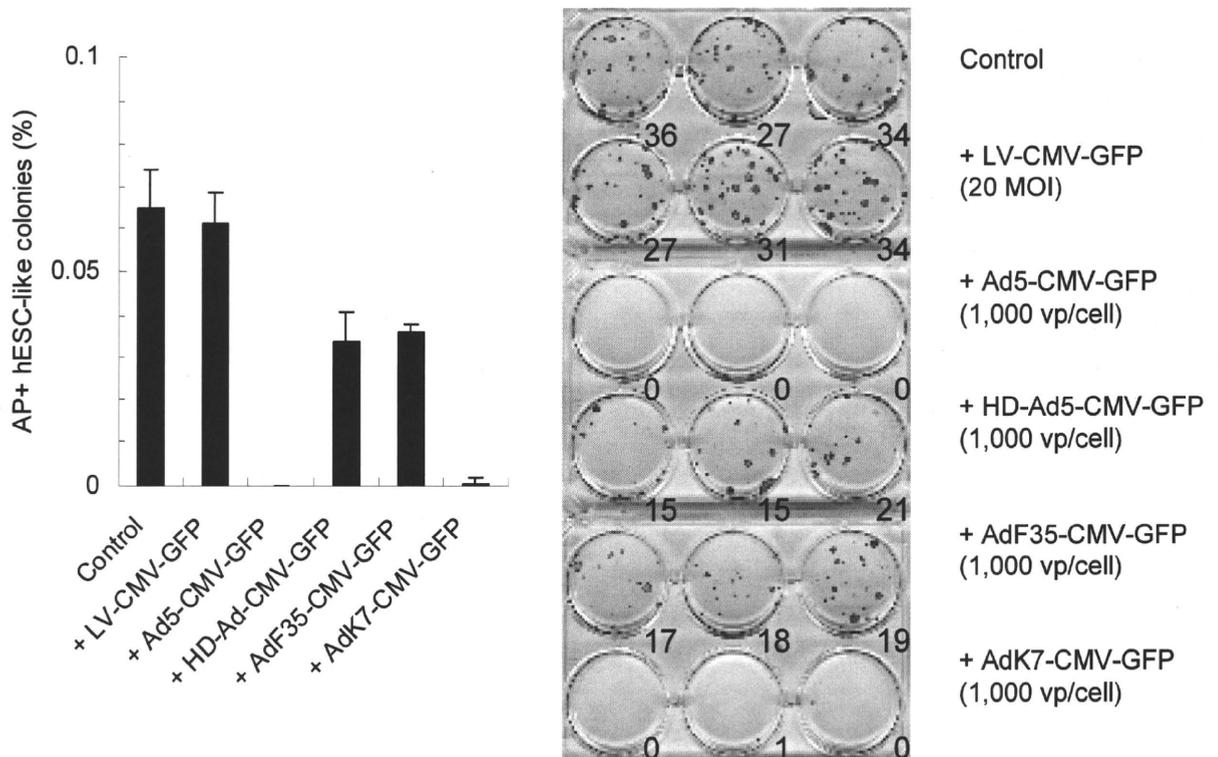
**Fig. 4 Effects of Ad vector transduction (low titer) on iPS cell generation**

(A) Experimental procedures. The procedures were the same as *Fig.3* except vector titer. (B) The number of AP-positive human ES cell-like colonies was counted and photographed.

(A)



(B)



**Fig. 5 Effects of Ad vector transduction (low titer) during iPS cell generation**

(A) Experimental procedures. HDF cells were transduced with lentiviral vectors expressing four reprogramming genes. Four days after lentiviral transduction, the cells were transduced with Ad vectors for 1.5h. Following procedures were the same as Fig.4. (B) The number of human ES cell-like colonies was counted and photographed.

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
川端健二、田代克久、 水口裕之	iPS 細胞への遺伝子導入を 用いた分化誘導の最適化	薬学雑誌	130	1527-1534	2010
Inamura M., Kawabata K., Takayama K., Tashiro K., Sakurai F., Katayama K., Toyoda M., Akutsu H., Miyagawa Y., Okita H., Kiyokawa N., Umezawa A., Hayakawa T., Furue MK., Mizuguchi H.	Efficient generation of hepatoblasts from human ES cells and iPS cells by transient overexpression of homeobox gene HEX.	Mol.Ther.,	Feb;19(2)	400-7	2010
Yohei Hayashi, Techuan Chan, Masaki Warashina, Masakazu Fukuda, Takashi Ariizumi, Koji Okabayashi, Naoya Takayama, Makoto Otsu, Koji Eto, Miho Kusuda Furue, Tatsuo Michiue, Kiyoshi Ohnuma, Hiromitsu Nakauchi, Makoto Asashima.	Reduction of N-glycolylneuraminic Acid in Human in Induced Pluripotent Stem Cells Generated or cultured under Feeder- and Serum-free Defined Conditions.	PLoS One	Nov23;5(11)	e14099	2010
Tashiro K., Kawabata K., Inamura M., Takayama K., Furukawa N., Sakurai F., Katayama K., Hayakawa H.,	Adenovirus vector-mediated efficient transduction into human embryonic and induced pluripotent stem cells.	Cell Reprogram.,	Oct;12(5):	501-7.	2010

Furue-Kusuda M., Mizuguchi H.,					
Yuko Aihara, Yohei Hayashi, Mitsuhi Hirata, Nobutaka Ariki, Shinsuke Shibata, Narihito Nagoshi, Mio Nakanishi, Kiyoshi Ohnuma, Masaki Warashina, Tatsuo Michiue, Hideho Uchiyama, Hideyuki Okano, Makoto Asashima and Miho Kusuda Furue	Induction of neural crest cells from mouse embryonic stem cells in a serum-free monolayer culture	Int. J. Dev. Biol.	54	1287 - 1294	2010
Jie Na, Miho K. Furue and Peter W. Andrews	Inhibition of ERK1/2 Prevents Neural and Mesendodermal Differentiation and Promotes Human Embryonic Stem Cell Self-renewal	Stem Cell Research	5(2)	157-69	2010
Miho Kusuda Furue, Daiki Tateyama, Masaki Kinehara, Jie Na, Tetsuji Okamoto, J. Denry Sato	Advantages and difficulties in culturing human pluripotent stem cells in growth factor-defined serum-free medium	In Vitro Cell Dev Biol Anim.	46	573-576	2010
Hayashi Y, Furue MK, Satoshi Tanaka, Michiko Hirose, Hiroki Danno, Kiyoshi Ohnuma, Shiho Oeda, Yuko Aihara, Kunio Shiota, Atsuo Ogura, Shoichi Ishiura, and Makoto Asashima	BMP4 Induces Trophoblast from Mouse Embryonic Stem Cells through the activation of Cdx2 expression.	In Vitro Cell Dev Biol Anim.	46	416-430	2010

S. Mimura, N. Kimura, M. Hirata, D. Tateyama, M. Hayashida, A. Umezawa, A. Kohara, H. Nikawa, T. Okamoto, M. Kusuda Furue	Growth Factor-Defined Culture Medium for Human Mesenchymal Stem Cells	Int. J. Dev. Biol.			2011
Toyoda M, Yamazaki-Inoue M, Itakura Y, Kuno A, Ogawa T, Yamada M, Akutsu H, Takahashi Y, Kanzaki S, Narimatsu H, Hirabayashi J, Umezawa A.	Lectin microarray analysis of pluripotent and multipotent stem cells.	Genes Cells	16(1)	1-11	2011
Higuchi A, Ling QD, Ko YA, Chang Y, Umezawa A.	Biomaterials for the Feeder-Free Culture of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells.	Chem Rev	<a href="https://doi.org/10.1021/cr1003612">dx.doi.org/10.1021/cr1003612</a> ,		2011
Nishino K, Toyoda M, Yamazaki-Inoue M, Makino H, Fukawatase Y, Chikazawa E, Takahashi Y, Miyagawa Y, Okita H, Kiyokawa N, Akutsu H, Umezawa A.	Defining hypo-methylated regions of stem cell-specific promoters in human iPS cells derived from extra-embryonic amnions and lung fibroblasts.	PLoS One	5(9)	e13017	2010
Sasaki N, Hirano T, Kobayashi K, Toyoda M, Miyakawa Y, Okita H, Kiyokawa N, Akutsu H, Umezawa A, Nishihara S.	Chemical inhibition of sulfation accelerates neural differentiation of mouse embryonic stem cells and human induced pluripotent stem cells.	Biochem Biophys Res Commun	401(3)	480-486	2010

Toyoda M, Hamatani T, Okada H, Matsumoto K, Saito H, Umezawa A.	Defining cell identity by comprehensive gene expression profiling.	Curr Med Chem	17(28)	3245-3252	2010
Adachi T, Wang X, Murata T, Obara M, Akutsu H, Machida M, Umezawa A, Tomita M.	Production of a non-triple helical collagen alpha chain in transgenic silkworms and its evaluation as a gelatin substitute for cell culture.	Biotechnol Bioeng	106(6)	860-870	2010
Fujino T, Nomura K, Ishikawa Y, Makino H, Umezawa A, Aburatani H, Nagasaki K, Nakamura T.	Function of EWS-POU5F1 in sarcomagenesis and tumor cell maintenance.	Am J Pathol	176(4)	1973-1982	2010

## iPS 細胞への遺伝子導入を用いた分化誘導の最適化

川端 健二,<sup>\*,a,b</sup> 田代 克久,<sup>a</sup> 水口 裕之<sup>a,c</sup>

## Differentiation of Functional Cells from iPS Cells by Efficient Gene Transfer

Kenji KAWABATA,<sup>\*,a,b</sup> Katsuhisa TASHIRO,<sup>a</sup> and Hiroyuki MIZUGUCHI<sup>a,c</sup>

<sup>a</sup>Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan, <sup>b</sup>Department of Biomedical Innovation and <sup>c</sup>Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

(Received July 20, 2010)

Induced pluripotent stem (iPS) cells, which are generated from somatic cells by transducing four genes, are expected to have broad application to regenerative medicine. Although establishment of an efficient gene transfer system for iPS cells is considered to be essential for differentiating them into functional cells, the detailed transduction characteristics of iPS cells have not been examined. By using an adenovirus (Ad) vector containing the cytomegalovirus enhancer/beta-actin (CA) promoters, we have developed an efficient transduction system for mouse mesenchymal stem cells and embryonic stem (ES) cells. Also, we applied our transduction system to mouse iPS cells and investigated whether efficient differentiation could be achieved by Ad vector-mediated transduction of a functional gene. As in the case of ES cells, the Ad vector could efficiently transduce transgenes into mouse iPS cells. We found that the CA promoter had potent transduction ability in iPS cells. Moreover, exogenous expression of a PPAR $\gamma$  gene or a Runx2 gene into mouse iPS cells by an optimized Ad vector enhanced adipocyte or osteoblast differentiation, respectively. These results suggest that Ad vector-mediated transient transduction is sufficient to promote cellular differentiation and that our transduction methods would be useful for therapeutic applications based on iPS cells.

**Key words**—regenerative medicine; induced pluripotent stem (iPS) cell; adenovirus vector; mesenchymal stem cell; embryonic stem (ES) cell

## 1. はじめに

幹細胞 (stem cells) は自己複製能と分化多能性という大きく2つの特徴を有する細胞であり、目的の細胞へ分化させることにより創薬や再生医療への応用が期待されている。再生医療への応用が期待されている幹細胞には、造血幹細胞、神経幹細胞、間葉系幹細胞、ES (embryonic stem) 細胞、iPS (induced pluripotent stem) 細胞などがある。このうち、ES 細胞は受精卵 (胚) から樹立され、iPS 細胞は体細胞に4種の遺伝子 (Oct-3/4, Sox2, Klf4, c-Myc) を導入することにより作製される。しかし

ながら、ES 細胞や iPS 細胞を直接生体に移植するには困難な場合も多く、マウス生体に投与するとランダムに分化してテラトーマ (奇形腫) を形成する。したがって、治療目的には幹細胞を *in vitro* で目的の細胞に分化させた後生体に移植することが望ましいと考えられる。幹細胞を骨、心筋、脂肪、血液などの目的の細胞に分化させるには、培養液に特定のサイトカインや増殖因子等の液性因子を加える方法がとられているが分化効率は十分ではない。そこで、われわれは各種幹細胞に機能遺伝子を導入することにより効率よく分化させることができないかと考え研究を進めている。一般に、幹細胞は遺伝子導入が困難であり、リポフェクション法やレトロウイルスベクター系など通常用いられる方法では十分な導入効率が得られない。われわれは高効率かつ一過性に目的遺伝子を発現させることができるアデノウイルス (Ad) ベクターを用いて機能遺伝子を導入

<sup>a</sup>独立行政法人医薬基盤研究所幹細胞制御プロジェクト (〒567-0085 大阪府茨木市彩都あさぎ7-6-8), <sup>b</sup>大阪大学大学院薬学研究科医薬基盤科学分野, <sup>c</sup>同分子生物学分野 (〒565-0871 大阪府吹田市山田丘1-6)

\*e-mail: kawabata@nibio.go.jp

本総説は、日本薬学会第130年会シンポジウムS19で発表したものを中心に記述したものである。

することにより、幹細胞の分化誘導効率を向上させることを目指した。

2. 間葉系幹細胞への遺伝子導入

間葉系幹細胞は骨髄由来のストローマ細胞であり、骨、軟骨、脂肪、心筋系列などの中胚葉系細胞に分化することができ、未分化状態で細胞を容易に増殖させることができる。<sup>1)</sup> また、最近では、間葉系幹細胞は神経細胞、肝細胞、インスリン産生細胞などの外胚葉や内胚葉系の細胞へも分化するという報告もあり、再生医療や組織工学への応用が強く期待されている。間葉系幹細胞の分化を制御する手段の1つとして、細胞分化に関与する遺伝子を導入することが挙げられる。Adベクターを用いた間葉系幹細胞への遺伝子導入も試みられてきたが、間葉系幹細胞はAd受容体CAR (coxsackievirus and adenovirus receptor) を発現していないためにその導入効率は極めて低く、遺伝子導入には高タイトーのベクターを必要としていた。<sup>2,3)</sup> われわれは、独自に開

発した種々のファイバー改変型Adベクターを用いて間葉系幹細胞にレポーター遺伝子を導入し、その発現効率を比較検討した。<sup>4,5)</sup> 間葉系幹細胞を多く含有する画分であるマウス骨髄ストローマ細胞を用いて遺伝子導入効率を測定した結果、間葉系幹細胞にはファイバーにポリリジン残基を挿入したK7型ベクターが最も適しており、従来型ベクターの100倍以上の遺伝子導入効率を示すことが明らかとなった [Figs. 1 (A) and (B)]. RGD型ベクターは従来型ベクターに比較し10倍程度の導入効率を示した。また、種々のプロモーターを用いて比較検討したところ、CAプロモーターが最適であった [Fig. 1 (C)]. したがって、間葉系幹細胞にはCAプロモーターを有するK7型Adベクターを用いることにより最も高効率に遺伝子導入できることが明らかとなった。間葉系幹細胞は様々な系列の細胞に分化するというだけでなく、担がんマウスに投与された場合には腫瘍に集積する性質を有している。<sup>6)</sup> し

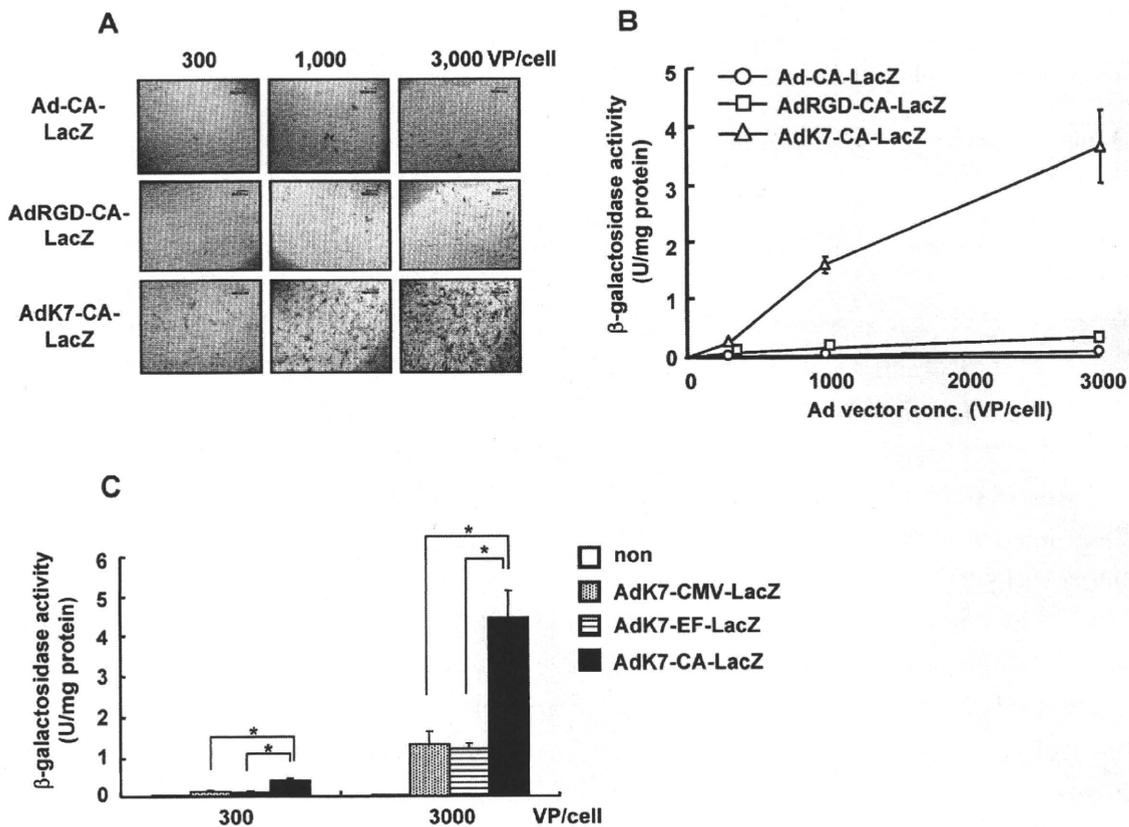


Fig. 1. Gene Transduction Efficiency in Mouse Primary BMSCs by Various Types of Ad Vectors

Mouse primary BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors for 1.5 h. Two days later, (A) X-gal staining and (B) luminescence assay were carried out. Similar results of X-gal staining were obtained in three independent experiments. Scale bar indicates 200 μm. (C) Optimization of promoter activity in BMSCs using LacZ-expressing AdK7. BMSCs were transduced with each Ad vector at 300 or 3000 VP/cell, and LacZ expression in the cells was measured. The data (B and C) are expressed as mean ± S.D. (n=3). \*p<0.01.

たがって、間葉系幹細胞は分化させた細胞自身を治療に利用するだけでなく、抗腫瘍性サイトカイン等を発現する間葉系幹細胞をがんに対する細胞治療薬として利用できる可能性があり、現在検討している。

### 3. 間葉系幹細胞の分化誘導

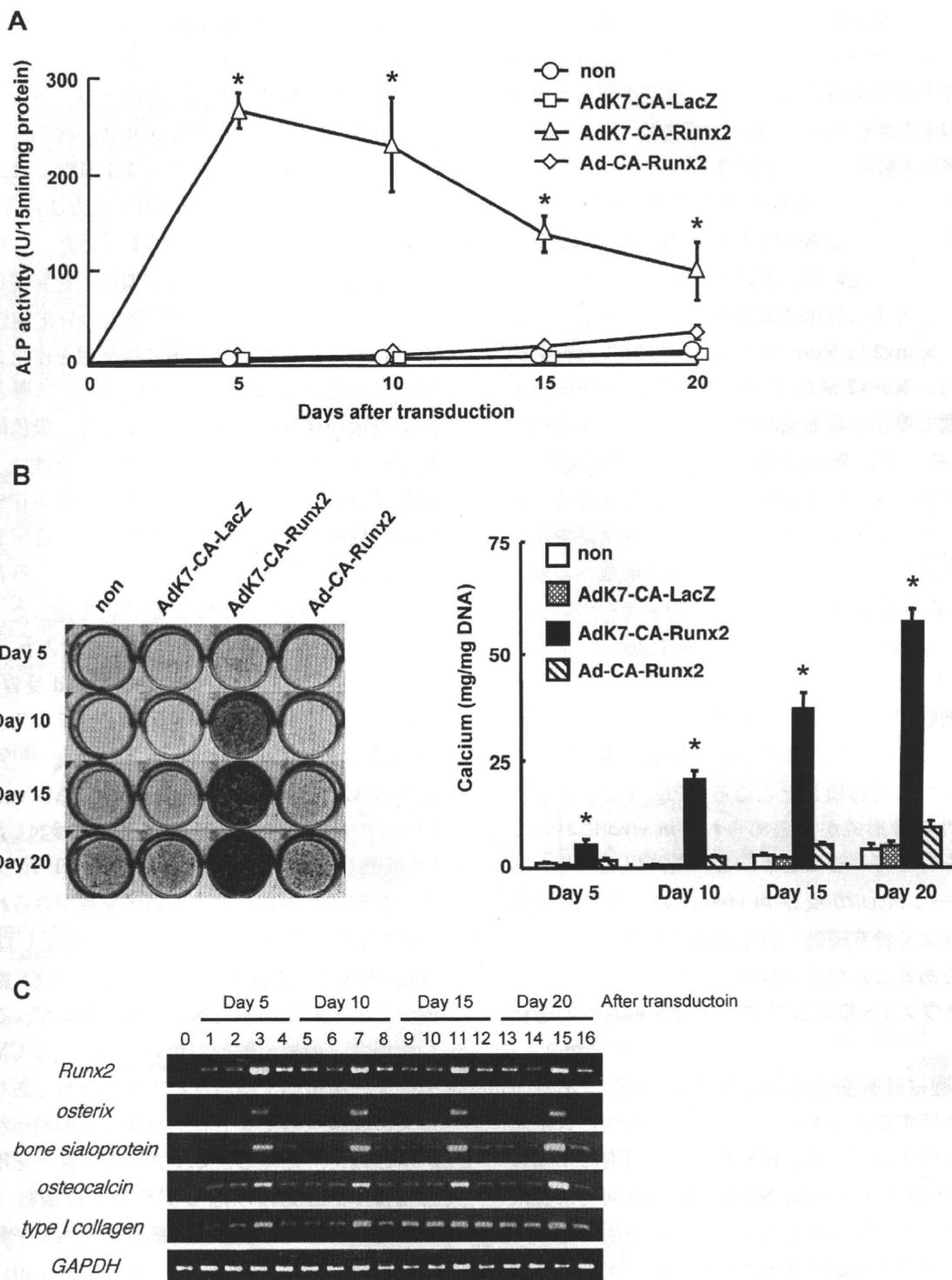
間葉系幹細胞への遺伝子導入には CA プロモーターを有した K7 型 Ad ベクターが最適であることが明らかとなったので、次に本ベクターを用いて機能遺伝子を導入し分化誘導効率を向上させることを試みた。Runx2 は Runt ファミリーに属する転写因子であり、Runx2 遺伝子欠損マウスは骨芽細胞分化が初期で停止し骨形成が欠損することが知られている。<sup>7)</sup>そこで、Runx2 遺伝子をマウス骨髄ストローマ細胞に導入した結果、従来の分化誘導法と比較しアルカリホスファターゼ活性の上昇及びカルシウムの沈着がみとめられ、著明に骨芽細胞への分化誘導効率が促進されることが明らかとなった [Figs. 2(A) and (B)]. また、それに伴い、タイプ I コラーゲン、オステリクス、オステオカルシン等の骨芽細胞マーカー遺伝子の発現も上昇した [Fig. 2(C)]. さらに、このようにして分化誘導した骨芽細胞をマウスに移植したところ、Fig. 3 に示すように異所性の骨形成がみとめられ、*in vivo* においても機能を有していることが示された。以上より、本ベクターは *in vitro* 及び *in vivo* において間葉系細胞を効率よく骨芽細胞へ分化誘導するのに適したベクターであることが示された。

### 4. マウス ES 細胞及びマウス iPS 細胞への遺伝子導入

ES 細胞は胚盤胞内部細胞塊由来の細胞であり、無限に増殖するとともにすべての機能細胞に分化する性質を有する。一方、iPS 細胞は体細胞に 4 種の遺伝子 (Oct-3/4, c-myc, Sox2, Klf4) を同時に導入することにより得られる人工多能性幹細胞であり、倫理的な問題を回避できることから再生医療への応用が大きく期待されている。<sup>8)</sup>しかしながら、これら幹細胞の分化を自由に制御する技術はいまだ確立されておらず、その原因の 1 つとして効率よい遺伝子導入法が確立されていないことが挙げられる。これまで、ES 細胞に対しては、プラスミド DNA を用いたエレクトポレーション法 (プラスミド DNA を電気的刺激により細胞内に導入し、染色体にわずかに目的遺伝子と薬剤耐性遺伝子が組み込まれた細

胞を薬剤で選択する方法)、<sup>9)</sup>レトロウイルスベクター、<sup>10)</sup>レンチウイルスベクター、<sup>11)</sup>ポリオーマウイルスの複製機構を利用したスーパーランスフェクション法 (ポリオーマウイルスの複製起点を含んだプラスミド DNA がマウス ES 細胞ではエピソードに増幅できる性質を利用した方法)<sup>12)</sup>などが外来遺伝子導入法として用いられてきた。しかしながら、これらは半永久的に導入遺伝子を発現し続ける方法であり、ES 細胞や iPS 細胞の分化制御、特に医療目的などの細胞分化後には発現を停止させたい場合には好ましくない。Ad ベクターは導入遺伝子が宿主染色体へ組込まれることなく、染色体外にエピゾームとして存在することから (増幅しない)、遺伝子発現が一過性であり、ES 細胞や iPS 細胞を目的の機能細胞に分化させた後は導入遺伝子の発現が消失するものと期待される。そこで、筆者らは、マウス ES 細胞及び iPS 細胞に最も適した Ad ベクターによる遺伝子導入法の確立を試みた。その結果、マウス ES 細胞や iPS 細胞は Ad 受容体 CAR を高発現しており、従来型アデノウイルスベクターが最適であることが明らかとなった。<sup>13,14)</sup>また、RSV, CMV, CA ( $\beta$ -actin promoter/CMV enhancer), EF-1 $\alpha$  の 4 種のプロモーターを用いて検討した結果、ES 細胞及び iPS 細胞では CA 及び EF-1 $\alpha$  プロモーターを用いた場合にのみ遺伝子発現がみられ、RSV や CMV プロモーターはほとんど機能しなかった [Fig. 4(A)]. これまで Ad ベクターは ES 細胞への遺伝子導入には不適と考えられてきたが、これは多くの場合、最も一般的に用いられている CMV プロモーターを用いて検討されてきたためであり、ウイルスの細胞へのエンタリー自体には問題がないことが示された。ただし、CA プロモーターを用いた場合には ES 細胞のみならずその支持細胞 (フィーダー細胞) である胚繊維芽細胞にも遺伝子発現がみられたのに対し、EF-1 $\alpha$  プロモーターを用いた場合にはほぼ ES 細胞特異的に遺伝子発現可能であった。これは、EF-1 $\alpha$  プロモーターの活性が胚繊維芽細胞に比べ ES 細胞において相対的に高いことが原因と考えられる。したがって、目的により両プロモーターを使い分けることによって、再生医療への幅広い応用が期待できる。

Ad ベクターを導入することにより ES 細胞や iPS 細胞が有する本来の性質が失われると再生医療



**Fig. 2. Promotion of *in Vitro* Osteoblastic Differentiation in BMSC Transduced with AdK7-CA-Runx2**

BMSCs were transduced with each Ad vector at 3000 VP/cell for 1.5 h, and were cultured for the indicated number of days. (A) Alkaline phosphatase activity, (B, left) matrix mineralization, and (B, right) calcium deposition in the cells was determined as described in Materials and Methods. The data are expressed as mean  $\pm$  S.D. ( $n=3$ ). \* $p<0.01$  as compared with non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells. (C) RT-PCR was carried out using primers for Runx2, osterix, bone sialoprotein, osteocalcin, collagen type I, and GAPDH. Lane 0: non-treated BMSCs; lanes 1, 5, 9, and 13: BMSCs with osteogenic supplements (OS); lanes 2, 6, 10, and 14: BMSCs with OS plus AdK7-CA-LacZ; lanes 3, 7, 11, and 15: BMSCs with OS plus AdK7-CA-Runx2; lanes 4, 8, 12, and 16: BMSCs with OS plus Ad-CA-Runx2.

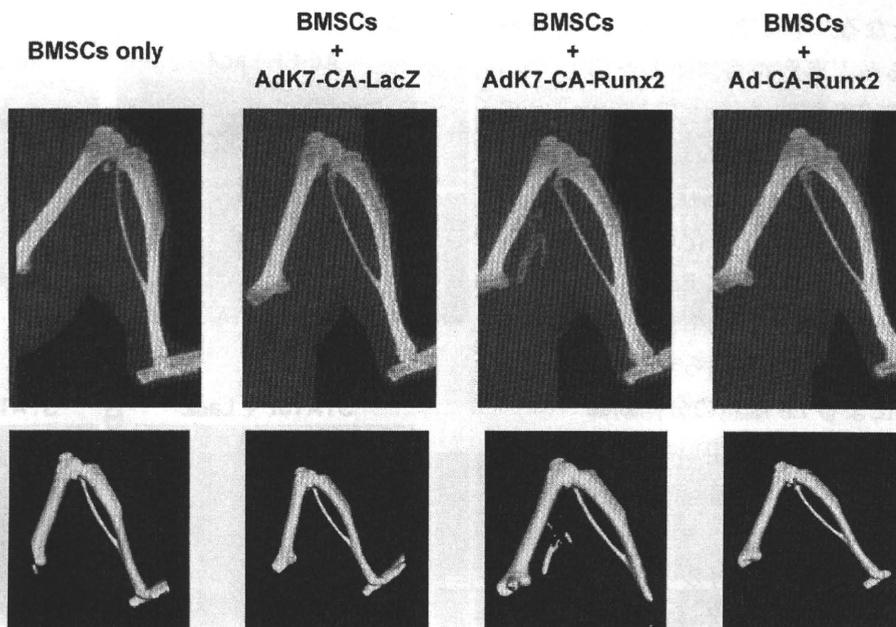


Fig. 3. *In vivo* Ectopic Bone Formation of Mouse BMSCs by AdK7-mediated Runx2 Gene Transduction

BMSCs were transduced with indicated Ad vectors at 3000 VP/cell for 1.5 h. On the following day, cells ( $2 \times 10^6$  cells) were suspended in PBS and injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by the microCT system. Similar results were obtained in two independent experiments. Upper, X-ray images; lower, 3D reconstitution images.

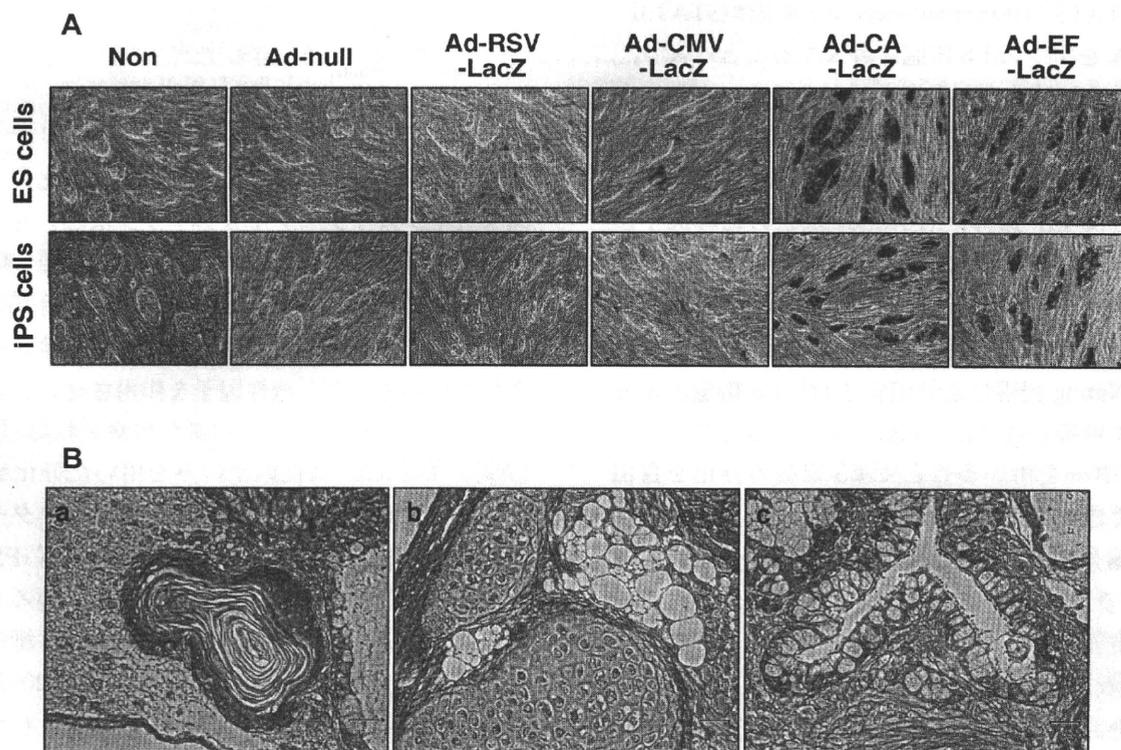


Fig. 4. Efficient Transgene Expression in Mouse iPS Cells by Using an Ad Vector Containing the CA and the EF-1 $\alpha$  Promoter

(A) Mouse ES cells or iPS cells were transduced with LacZ-expressing Ad vector at 3000 VP/cell. On the following day, X-gal staining was carried out. Similar results of X-gal staining were obtained in three independent experiments. (B) Paraffin sections of the teratomas derived from Ad-CA-mCherry-transduced iPS cells were prepared, and sections were stained with hematoxylin and eosin. a, ectoderm (epidermis); b, mesoderm (cartilage and adipocyte); c, endoderm (gut epithelium).

への応用は困難となる。そこで、三胚葉への分化能が保持されているかどうかを検討するために、Adベクター導入後のiPS細胞を用いてテラトーマ形成実験を行った [Fig. 4(B)]。その結果、遺伝子導入されたiPS細胞はもとのiPS細胞と同様に外胚葉・中胚葉・内胚葉いずれにも分化可能であることが明らかとなり、AdベクターはES細胞やiPS細胞の分化能を妨げることなく、効率よく遺伝子導入ができるベクターであることが明らかとなった。

#### 5. 遺伝子導入によるES細胞の分化制御

最適化されたAdベクターを用いてES細胞に機能遺伝子を導入し、実際にES細胞の分化を制御できるかどうかについて検討した。マウスES細胞はフィーダー細胞由来のサイトカインLIF (leukemia inhibitory factor) がその未分化維持に必須であることが知られている。LIFは受容体に結合後、下流のSTAT3 (signal transducer and activator of transcription 3) を介してシグナルを伝達する。そこで、EF-1 $\alpha$ プロモーターを有した従来型Adベクターを用いてSTAT3のdominant-negative変異体(STAT3F)のcDNAをマウスES細胞に導入することにより、LIFの下流シグナルを阻害させたと、LIF存在下でもES細胞は三胚葉すべての細胞に分化することが明らかとなった [Fig. 5(A)]。ES細胞の未分化維持にはLIF以外にもNanogなどの転写因子が必須であることが明らかとなっている。そこで、前述のベクターを用いてSTAT3FとNanogを同時に発現させたと、STAT3Fによる細胞分化シグナルがNanog発現により阻害され、ES細胞は未分化状態を維持し続けた [Fig. 5(B)]。したがって、Adベクターを用いることでES細胞の分化を自由に制御できる可能性が示された。

#### 6. ES細胞及びiPS細胞の高効率分化誘導

Adベクターを用いた遺伝子導入技術が分化誘導系に応用できるかどうかを検討するために、マウスES細胞及びiPS細胞へ機能遺伝子を導入することを試みた。ES細胞及びiPS細胞からの分化モデルとして脂肪細胞への分化誘導を行うとともに、脂肪細胞分化に必須の遺伝子であるPPAR $\gamma$  (peroxisome proliferator-activated receptor gamma) 遺伝子<sup>15)</sup>をES細胞及びiPS細胞へ導入することにより、脂肪細胞への分化効率が上昇するかどうかを検討した。両細胞へLacZ遺伝子(コントロール)又

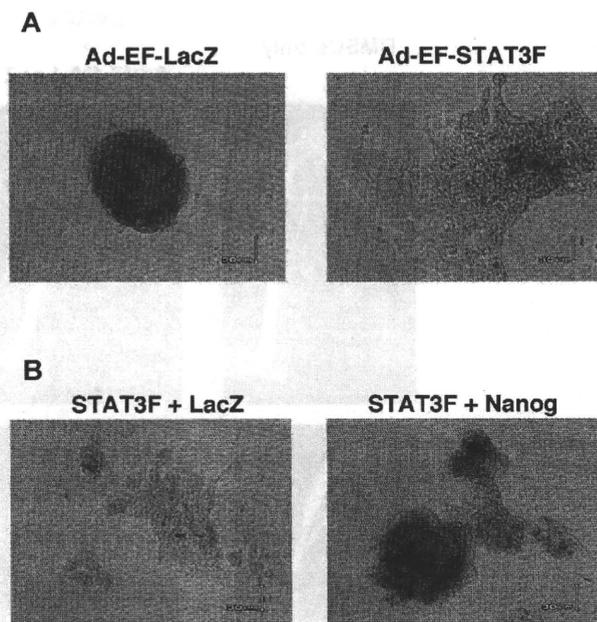


Fig. 5. Introduction of Functional Genes into Mouse ES Cells by Ad Vectors Containing EF-1 $\alpha$  Promoter

Mouse ES cells ( $1 \times 10^4$  cells) were seeded, and on the following day, the cells were transduced with 3000 VP/cell of Ad-EF-LacZ or Ad-EF-STAT3F for 1.5 h (A). Mouse ES cells were also co-infected with 3000 VP/cell of Ad-EF-STAT3F and 3000 VP/cell of Ad-EF-lacZ or Ad-EF-Nanog for 1.5 h (B). On day 3, each cell was infected again by the same vectors. On day 5, alkaline phosphatase staining was performed. Alkaline phosphatase-positive cells indicate undifferentiated ES cells. Similar results were obtained in three independent experiments.

はPPAR $\gamma$ 遺伝子を導入し、脂肪細胞分化用の液性因子(インスリン、デキサメタゾンなど)を含む培地中で15日間接着培養した。オイルレッドO染色により脂肪細胞への分化効率を評価した結果、iPS細胞の脂肪細胞への分化効率はES細胞の分化効率よりも低いものの、液性因子を作用させて培養することにより両細胞とも脂肪滴が観察された [Fig. 6(A)]。さらに、液性因子のみを用いた分化誘導法と比較し、液性因子を加えさらにAdベクターによるPPAR $\gamma$ 遺伝子を導入したES細胞及びiPS細胞は、極めて効率よく脂肪細胞へ分化していることも示された。すなわち、従来の方法ではES細胞において約50%の細胞が、iPS細胞において20-30%の細胞がそれぞれオイルレッドOで染色されたのに対し、PPAR $\gamma$ 遺伝子を導入したES細胞及びiPS細胞においては80-90%の細胞がオイルレッドOに染色された。また、Adベクターを用いたPPAR $\gamma$ 遺伝子導入による脂肪細胞への分化効率の上昇は、脂肪細胞特異的なマーカー遺伝子の発現上昇によっても確認された。なお、LacZ遺伝子を導

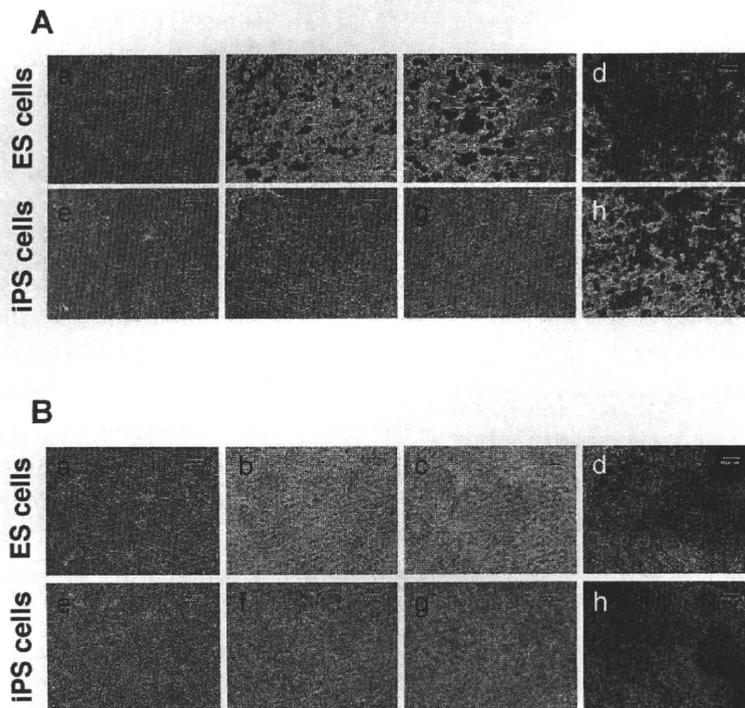


Fig. 6. Efficient Adipocytic or Osteoblastic Differentiation from Mouse ES Cells and iPS Cells by the Transduction of the PPAR $\gamma$  or Runx2 Gene

(A) ES-EBs or iPS-EBs were transduced in triplicate with 10000 VP/cell of Ad-CA-LacZ or Ad-CA-PPAR $\gamma$ . After plating onto a gelatin-coated dish on day 7, ES-EBs and iPS-EBs were cultured for 15 days in the presence or absence of adipogenic supplements (AS). After cultivation, lipid accumulation was detected by oil red O staining. a, non-treated ES-EBs; b, ES-EBs with AS; c, ES-EBs with AS plus Ad-CA-LacZ; d, ES-EBs with AS plus Ad-CA-PPAR $\gamma$ ; e, non-treated iPS-EBs; f, iPS-EBs with AS; g, iPS-EBs with AS plus Ad-CA-LacZ; h, iPS-EBs with AS plus Ad-CA-PPAR $\gamma$ . The scale bar indicates 60  $\mu$ m. (B) ES-EBs or iPS-EBs were transduced in triplicate with 10000 VP/cell of Ad-CA-LacZ or Ad-CA-Runx2. After culturing for 15 days with or without osteogenic supplements (OS), matrix mineralization in the cells was detected by von Kossa staining. a, non-treated ES-EBs; b, ES-EBs with OS; c, ES-EBs with OS plus Ad-CA-LacZ; d, ES-EBs with OS plus Ad-CA-Runx2; e, non-treated iPS-EBs; f, iPS-EBs with OS; g, iPS-EBs with OS plus Ad-CA-LacZ; h, iPS-EBs with OS plus Ad-CA-Runx2. The scale bar indicates 60  $\mu$ m.

入した細胞ではこのような分化効率の上昇はみとめられなかった。これらの結果から、マウス ES 細胞及び iPS 細胞の脂肪細胞への分化効率は Ad ベクターを用いた PPAR $\gamma$  遺伝子の導入により改善できることが示された。

次に、Ad ベクターによる遺伝子導入がその他の細胞種への分化誘導系にも有用であるかどうかを検討するため、マウス ES 細胞及び iPS 細胞から骨芽細胞への分化誘導を試みた。今回の分化誘導系においては前述した Runx2 遺伝子を Ad ベクターにより発現させた。Ad-CA-Runx2 を EB (embryoid body) へ 3 回作用させ、骨芽細胞分化用の液性因子 ( $\beta$  グリセロリン酸, アスコルビン酸など) 中で接着培養し、マウス ES 細胞及び iPS 細胞由来の細胞が石灰化を生ずる骨芽細胞へ分化しているかどうかを von Kossa 染色により解析した。その結果、液性因子を作用させることにより石灰化が検出されたものの、その分化効率は低く、20% 以下の細胞し

か石灰化を起こしていなかった [Fig. 6(B)]。一方、Runx2 遺伝子を導入した ES 細胞及び iPS 細胞は、液性因子のみで培養した細胞及び LacZ 遺伝子を導入した細胞と比較し、石灰化した細胞が著明に増加していること明らかとなった [Fig. 6(B)]。また、沈着した石灰化を定量化したところ、LacZ 遺伝子導入群においては液性因子のみの誘導法と著差はみとめられなかったものの、Runx2 遺伝子導入細胞においては約 8 倍、石灰化が上昇していた。これらの結果から、Ad ベクターによる Runx2 遺伝子の導入によりマウス ES 細胞及び iPS 細胞から骨芽細胞へ効率よく分化誘導可能であることが示された。このように最適化した Ad ベクターを用いたマウス ES 細胞及び iPS 細胞への分化関連遺伝子の導入により、脂肪細胞及び骨芽細胞への分化効率を飛躍的に改善できることが示され、本遺伝子導入技術はマウス ES 細胞及び iPS 細胞を用いた細胞分化研究に有用であると考えられた。

## 7. おわりに

これまでもレトロウイルスベクターなどの恒常的遺伝子発現系を用いて ES 細胞から標的の細胞への分化誘導は行われてきた。しかしながら、これらの手法では機能遺伝子が染色体に挿入されるため治療用に適しておらず、一過性の遺伝子導入系の分化誘導が望まれていた。今回、われわれは Ad ベクターを用いたマウス ES 細胞及び iPS 細胞への高効率遺伝子導入法を確立し、さらにその遺伝子導入技術を利用して分化関連遺伝子をマウス ES 細胞及び iPS 細胞へ導入することにより特定の細胞へ効率よく分化誘導することに成功した。なお、異なる iPS 細胞株についても今回と同様の結果が得られており、<sup>14)</sup> 幅広い ES 細胞及び iPS 細胞株に適用可能であることが示唆されている。また、今回は示していないが、分化が完了した細胞では Ad ベクター由来の遺伝子発現はほとんどみとめられないことも確認している。<sup>16)</sup> したがって、Ad ベクターは幹細胞を用いた分化誘導研究において、効率面だけでなく安全面においても非常に有用であると考えられる。現在、筆者らのグループでは Ad ベクターを用いた遺伝子導入技術がその他の細胞種への分化誘導系へ応用可能かどうかに関して、マウス及びヒトの ES 細胞及び iPS 細胞を用いて検討中である。一過性発現を示す Ad ベクターを用いた遺伝子導入技術は、幹細胞研究・再生医療研究において重要なツールになるものと考えられ、今後のますますの応用が期待される。

## REFERENCES

- 1) Pittenger M. F., Mackay A. M., Beck S. C., Jaiswal R. K., Douglas R., Mosca J. D., Moorman M. A., Simonetti D. W., Craig S., Marshak D. R., *Science*, **284**, 143-147 (1999).
- 2) Conget P. A., Minguell J. J., *Exp. Hematol.*, **28**, 382-390 (2000).
- 3) Hung S. C., Lu C. Y., Shyue S. K., Liu H. C., Ho L. L., *Stem Cells*, **22**, 1321-1329 (2004).
- 4) Mizuguchi H., Sasaki T., Kawabata K., Sakurai F., Hayakawa T., *Biochem. Biophys. Res. Commun.*, **332**, 1101-1106 (2005).
- 5) Tashiro K., Kondo A., Kawabata K., Sakurai H., Sakurai F., Yamanishi K., Hayakawa T., Mizuguchi H., *Biochem. Biophys. Res. Commun.*, **30**, 127-132 (2009).
- 6) Studeny M., Marini F. C., Dembinski J. L., Zompetta C., Cabreira-Hansen M., Bekele B. N., Champlin R. E., Andreeff M., *J. Natl. Cancer Inst.*, **96**, 1593-1603 (2004).
- 7) Komori T., *J. Cell. Biochem.*, **95**, 445-453 (2005).
- 8) Takahashi K., Yamanaka S., *Cell*, **126**, 663-676 (2006).
- 9) Tompers D. M., Labosky P. A., *Stem Cells*, **22**, 243-249 (2004).
- 10) Cherry S. R., Biniszkiwicz D., van Parijs L., Baltimore D., Jaenisch R., *Mol. Cell. Biol.*, **20**, 7419-7426 (2000).
- 11) Gropp M., Itsykson P., Singer O., Ben-Hur T., Reinhartz E., Galun E., Reubinoff B. E., *Mol. Ther.*, **7**, 281-287 (2003).
- 12) Niwa H., Masui S., Chambers I., Smith A. G., Miyazaki J., *Mol. Cell. Biol.*, **22**, 1526-1536 (2002).
- 13) Kawabata K., Sakurai F., Yamaguchi T., Hayakawa T., Mizuguchi H., *Mol. Ther.*, **12**, 547-554 (2005).
- 14) Tashiro K., Inamura M., Kawabata K., Sakurai F., Yamanishi K., Hayakawa T., Mizuguchi H., *Stem Cells*, **27**, 1802-1811 (2009).
- 15) Tontonoz P., Hu E., Spiegelman B. M., *Cell*, **79**, 1147-1156 (1994).
- 16) Tashiro K., Kawabata K., Sakurai H., Kurachi S., Sakurai F., Yamanishi K., Mizuguchi H., *J. Gene Med.*, **10**, 498-507 (2008).

# Efficient Generation of Hepatoblasts From Human ES Cells and iPS Cells by Transient Overexpression of Homeobox Gene *HEX*

Mitsuru Inamura<sup>1,2</sup>, Kenji Kawabata<sup>2,3</sup>, Kazuo Takayama<sup>1,2</sup>, Katsuhisa Tashiro<sup>2</sup>, Fuminori Sakurai<sup>2</sup>, Kazufumi Katayama<sup>1,2</sup>, Masashi Toyoda<sup>4</sup>, Hidenori Akutsu<sup>4</sup>, Yoshitaka Miyagawa<sup>5</sup>, Hajime Okita<sup>5</sup>, Nobutaka Kiyokawa<sup>5</sup>, Akihiro Umezawa<sup>4</sup>, Takao Hayakawa<sup>6,7</sup>, Miho K Furue<sup>8,9</sup> and Hiroyuki Mizuguchi<sup>1,2</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan;

<sup>2</sup>Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka, Japan; <sup>3</sup>Department of Biomedical Innovation, Graduate School of Pharmaceutical Science, Osaka University, Osaka, Japan; <sup>4</sup>Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo, Japan; <sup>5</sup>Department of Developmental Biology and Pathology, National Institute for Child Health and Development, Tokyo, Japan; <sup>6</sup>Pharmaceuticals and Medical Devices Agency, Tokyo, Japan; <sup>7</sup>Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan; <sup>8</sup>JCRB Cell Bank/Laboratory of Cell Culture, Department of Disease Bioresource, National Institute of Biomedical Innovation, Osaka, Japan; <sup>9</sup>Laboratory of Cell Processing, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into all cell lineages, including hepatocytes, *in vitro*. Induced hepatocytes have a wide range of potential application in biomedical research, drug discovery, and the treatment of liver disease. However, the existing protocols for hepatic differentiation of PSCs are not very efficient. In this study, we developed an efficient method to induce hepatoblasts, which are progenitors of hepatocytes, from human ESCs and iPSCs by overexpression of the *HEX* gene, which is a homeotic gene and also essential for hepatic differentiation, using a *HEX*-expressing adenovirus (Ad) vector under serum/feeder cell-free chemically defined conditions. Ad-*HEX*-transduced cells expressed  $\alpha$ -fetoprotein (AFP) at day 9 and then expressed albumin (ALB) at day 12. Furthermore, the Ad-*HEX*-transduced cells derived from human iPSCs also produced several cytochrome P450 (CYP) isozymes, and these P450 isozymes were capable of converting the substrates to metabolites and responding to the chemical stimulation. Our differentiation protocol using Ad vector-mediated transient *HEX* transduction under chemically defined conditions efficiently generates hepatoblasts from human ESCs and iPSCs. Thus, our methods would be useful for not only drug screening but also therapeutic applications.

Received 18 March 2010; accepted 13 October 2010; published online 23 November 2010. doi:10.1038/mt.2010.241

## INTRODUCTION

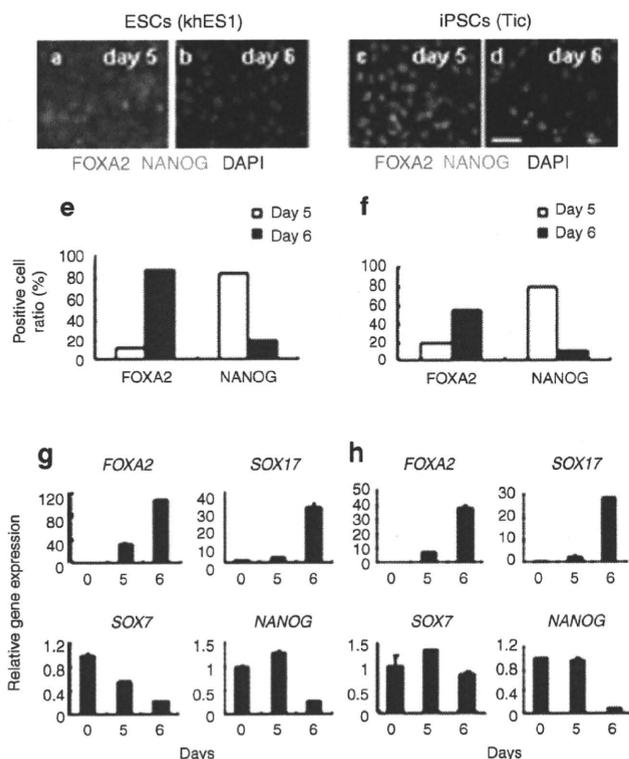
Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body,<sup>1-4</sup> and thereby have the potential to provide an unlimited source of cells for a variety of

applications.<sup>5</sup> Hepatocytes are useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly applicable to drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models, because the liver is the main detoxification organ in the body.<sup>6</sup> For these applications, it is necessary to prepare a large number of functional hepatocytes from human ESCs and iPSCs. Many of the existing methods for cell differentiation of human ESCs and iPSCs into hepatocytes employ undefined, serum-containing medium and feeder cells.<sup>7-9</sup> Preparation of human ESC- and iPSC-derived hepatocytes for therapeutic applications and drug toxicity testing in humans should be done in nonxenogenic culture systems to avoid potential contamination with pathogens. Furthermore, the efficiency of the differentiation of the human ESCs and iPSCs into hepatocytes is not particularly high using these methods.<sup>9-14</sup>

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm.<sup>5,15</sup> Shortly afterwards, the definitive endoderm is separated into endoderm derivatives containing the liver bud, the cells of which are referred to as hepatoblasts. The hepatoblasts have the potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In the process of hepatic differentiation, the maturation is characterized by the expression of liver- and stage-specific genes. For example,  $\alpha$ -fetoprotein (AFP) is an early hepatic marker, which is expressed in hepatoblasts in the liver bud until birth, and its expression is dramatically reduced after birth.<sup>16</sup> In contrast, albumin (ALB), which is the most abundant protein synthesized by hepatocytes, is initially expressed at lower levels in early fetal hepatocytes, but its expression level is increased as the hepatocytes mature, reaching a maximum in adult hepatocytes.<sup>17</sup> Furthermore, isoforms of cytochrome P450 (CYP) proteins also exhibit differential expression levels according to the developmental stages

**Correspondence:** Hiroyuki Mizuguchi, Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: mizuguch@phs.osaka-u.ac.jp





**Figure 2** Characterization of the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. (a–d) The immunofluorescent staining of the human ESC (khES1)- and iPSC (Tic) derived differentiated cells before (a and c; day 5) and after passaging (b and d; day 6). The cells were immunostained with antibodies against FOXA2 and NANOG. Nuclei were stained with DAPI. (e,f) Semiquantitative analysis of the immunofluorescent staining in a–d. Data are presented as the mean of immunopositive cells counted in eight independent fields. (g,h) Real-time RT-PCR analysis of the level of definitive endoderm (FOXA2 and SOX17), pluripotent (NANOG), and extra-embryonic endoderm (SOX7) gene expression at day 5 and 6. At day 5, the cells were passaged. Therefore, the data at day 5 and 6 show the levels of gene expression before (at day 5) or after the passage (at day 6). Data are presented as the mean  $\pm$  SD from triplicate experiments. The graphs represent the relative gene expression level when the level of undifferentiated cells at day 0 was taken as 1. Bar = 50  $\mu$ m. ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

## RESULTS

### Differentiation of human ESC- and iPSC-derived definitive endoderms

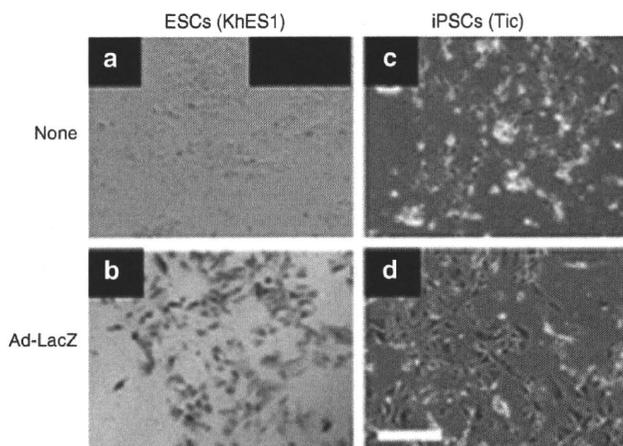
Our three-step differentiation protocol is illustrated in Figure 1a. After treatment with 50 ng/ml of Activin A (high-dose) and basic fibroblast growth factor (bFGF) for 5 days on a laminin-coated plate, morphologically, the human ESCs and iPSCs were gradually transformed from typical, defined, tight human ESC, and iPSC colonies (day 0) into less dense, flatter cells containing prominent nuclei (day 5), even though the majority of the cells had a morphology resembling that of undifferentiated cells (Figure 1b,c,f,g). FACS analysis showed that ~46% of human iPSC-derived differentiated cells expressed CXCR4 (expressed in the definitive endoderm but not the primitive endoderm) (Supplementary Figure S1a). Human ESC- and iPSC-derived differentiated cells were immunostained with the definitive endoderm marker, FOXA2 (Figure 2a,c). However, the majority of the cells expressed the pluripotent marker NANOG, indicating that undifferentiated

cells remain in the induced cultures at day 5. After the cells were passaged with trypsin-EDTA and seeded on a laminin-coated plate a second time, the resultant cells were found to be more homogeneous and flatter at day 6 (Figure 1d,h). Semiquantitative analysis by counting immunopositive cells revealed that the number of FOXA2-positive cells was increased and, in turn, the number of NANOG-positive cells was decreased at day 6 after passaging (Figure 2e,f). Real-time reverse transcriptase (RT)-PCR analysis showed that the definitive endoderm markers FOXA2 and SOX17 mRNA were upregulated, whereas the pluripotent marker NANOG mRNA was downregulated at day 6 (Figure 2g,h). These results were consistent with the immunofluorescence results (Figure 2a–d). The expression levels of the mesoderm marker *FLK1* mRNA and ectoderm marker *PAX6* mRNA were downregulated or unchanged at day 6 (Supplementary Figure S1b–e). Importantly, the expression of SOX7 mRNA (expressed in the extra-embryonic endoderm but not the definitive endoderm) was downregulated (Figure 2g,h). These results indicate that the definitive endoderm is induced or selected from human ESCs and iPSCs after passaging. We obtained the same results using another human iPSC line (Supplementary Figure S2a–d).

### HEX induces hepatoblasts from the human ESC- and iPSC-derived definitive endoderms

To investigate whether forced expression of transcription factors could promote hepatic differentiation, the human ESC- and iPSC-derived definitive endoderms were transduced with Ad vectors. We used a fiber-modified Ad vector containing the elongation factor-1 $\alpha$  promoter and a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob to examine the transduction efficiency in the human ESC- and iPSC-derived definitive endoderms. The elongation factor-1 $\alpha$  promoter was found to be highly active in human ESCs.<sup>28</sup> The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing K7 peptides was shown to be efficient for transduction into many kinds of cells.<sup>29,30</sup> The human ESC- and iPSC-derived definitive endoderms were transduced with a LacZ-expressing Ad vector (Ad-LacZ) at 3,000 vector particle/cell. X-Gal staining showed that the Ad-LacZ-transduced human ESC- and iPSC-derived definitive endoderms successfully expressed LacZ (Figure 3). Nearly 100% of the cells transduced with Ad-LacZ were strongly X-gal positive. The transduction efficiency in the human ESC- and iPSC-derived definitive endoderms transduced with the conventional Ad vector containing the wild-type capsid at 3,000 vector particle/cell was ~80% and X-gal staining was much weaker than that in the cells transduced with fiber-modified Ad vectors (Supplementary Figure S6).

Next, the human ESC- and iPSC-derived definitive endoderms were transduced with a HEX-expressing fiber-modified Ad vector (Ad-HEX). Although HEX is known to be a transcription factor that is essential for liver development, it remains unclear what the effect of transient *HEX* overexpression is on differentiation from human ESCs and iPSCs or their derivatives *in vitro*. We confirmed the overexpression of *HEX* in the human ESC- and iPSC-derived definitive endoderms transduced with Ad-HEX (Supplementary Figure S3a–f). Gene expression analysis revealed the upregulation of *AFP* mRNA, which was expressed by hepatoblasts or early hepatocytes, in Ad-HEX-transduced cells as



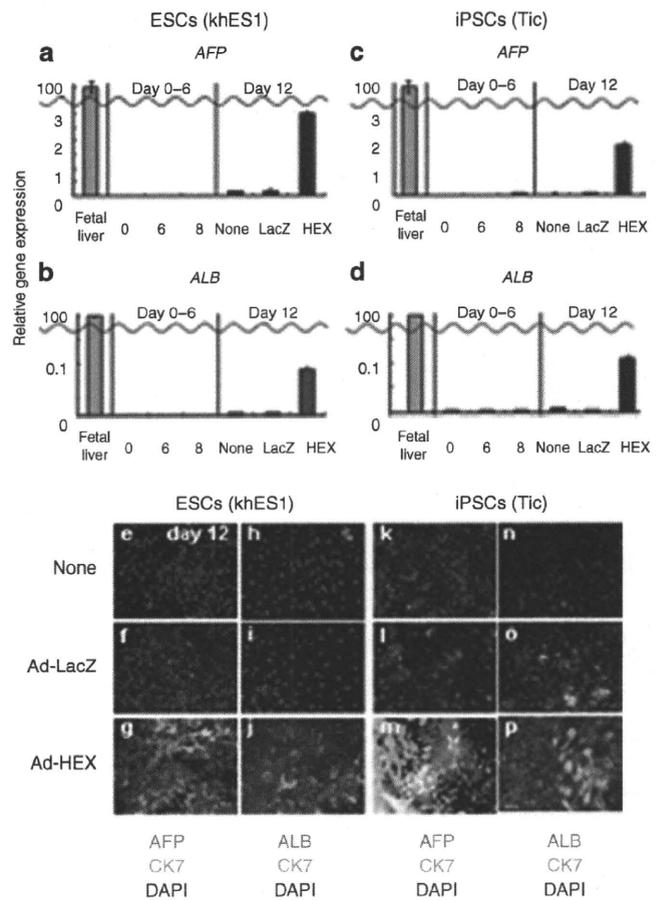
**Figure 3** Efficient transgene expression in the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by using a fiber-modified Ad vector containing the EF-1 $\alpha$  promoter. (a,b) Human ESC (khES1)-derived and (c,d) iPSC (Tic) derived definitive endoderms were transduced with 3,000VP/cell of Ad-LacZ for 1.5 hours. The next day after transduction, X-gal staining was performed as described in the Materials and Methods section. Similar results were obtained in two independent experiments. Scale = 50 $\mu$ m. Ad, adenovirus; EF-1 $\alpha$ , elongation factor-1 $\alpha$ ; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

compared with nontransduced cells or Ad-LacZ-transduced cells (Figure 4a,c). Expression of ALB mRNA, which is the most abundant protein in liver, was also observed in Ad-HEX-transduced cells (Figure 4b,d).

During liver development, both hepatocytes and cholangiocytes were differentiated from the hepatoblasts. We examined the protein expression of AFP, ALB, and the cholangiocyte marker cytokeratin 7 (CK7) in Ad-HEX-transduced cells by immunostaining (Figure 4e–p). The AFP-positive populations were detected in Ad-HEX-transduced cells (Figure 4g,m). ALB-positive cells were also detected, although the detection efficiency was very low (Figure 4j,p). CK7-positive cells were observed among the Ad-HEX-transduced cells, and all CK7-positive cells were found near the AFP- and ALB-positive cells, suggesting that hepatoblasts are generated by the transient overexpression of a *HEX* gene. Semiquantitative RT-PCR analysis showed that the expression levels of the liver-enriched transcription factors hepatocyte nuclear factor 1A, hepatocyte nuclear factor 1B, hepatocyte nuclear factor 4A, and hepatocyte nuclear factor 6 mRNA were upregulated in Ad-HEX-transduced cells (Supplementary Figure S4a,b). The expressions of CCAAT/enhancer binding protein  $\alpha$  and prospero homeobox 1 mRNA, two transcription factors known to play a pivotal role in the establishment of the hepatoblasts, were also induced in Ad-HEX-transduced cells (Supplementary Figure S4a, b). Taken together, these findings indicate that *HEX* enhances the specification of hepatoblasts from the human ESC- and iPSC-derived definitive endoderms. Similar results were obtained with another human iPSC line (Supplementary Figure S2e–g).

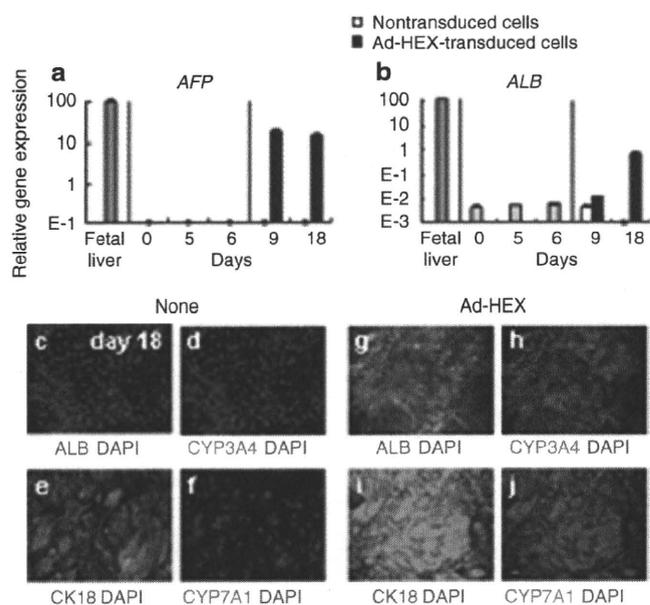
### Time course of differentiation of the definitive endoderm to hepatoblasts

Next, we examined the time course of AFP and CK7 expression during differentiation of human iPSCs to hepatoblasts in Ad-HEX-



**Figure 4** Efficient hepatoblast differentiation from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms by transduction of the *HEX* gene. (a–d) Real-time RT-PCR analysis of the level of (a,c) *AFP* and (b,d) *ALB* expression in nontransduced cells, Ad-LacZ-transduced cells, and Ad-HEX-transduced cells, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms (day 0, 5, 6, and 12). The cells were transduced with Ad-LacZ or Ad-HEX at day 6 as described in Figure 1a. The data at day 6 was obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression levels when the level in the fetal liver was taken as 100. (e–p) Immunocytochemistry of AFP, ALB, and CK7 expression in nontransduced cells (e,h,k, and n), Ad-LacZ-transduced cells (f,i,l, and o), and Ad-HEX-transduced cells (g,j,m, and p) at day 12, all of which were induced from the human ESC (khES1)- and iPSC (Tic) derived definitive endoderms. Nuclei were stained with DAPI. Bar = 50 $\mu$ m. Ad, adenovirus; AFP,  $\alpha$ -fetoprotein; ALB, albumin; CK7, cytokeratin 7; HEX, Ad-HEX-transduced cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

transduced cells and nontransduced cells. At day 7 (the day after transduction), the expression of AFP was not detectable in Ad-HEX-transduced or nontransduced cells (Supplementary Figure S5a,d). At day 8–9, morphological changes to hepatocyte-like cells were observed in Ad-HEX-transduced cells (Supplementary Figure S5h,i). We also observed homogeneous AFP-positive cells at day 9 (Supplementary Figure S5e). At day 10, CK7-positive cells appeared, indicating that hepatoblasts started to differentiate into hepatocytes and cholangiocytes at day 9–10 (Supplementary Figure S5f). At day 12, ALB-positive cells appeared, indicating that hepatocytes were differentiated from Ad-HEX-transduced cells (Figure 4p). These results showed that *HEX* induces the hepatoblasts from the

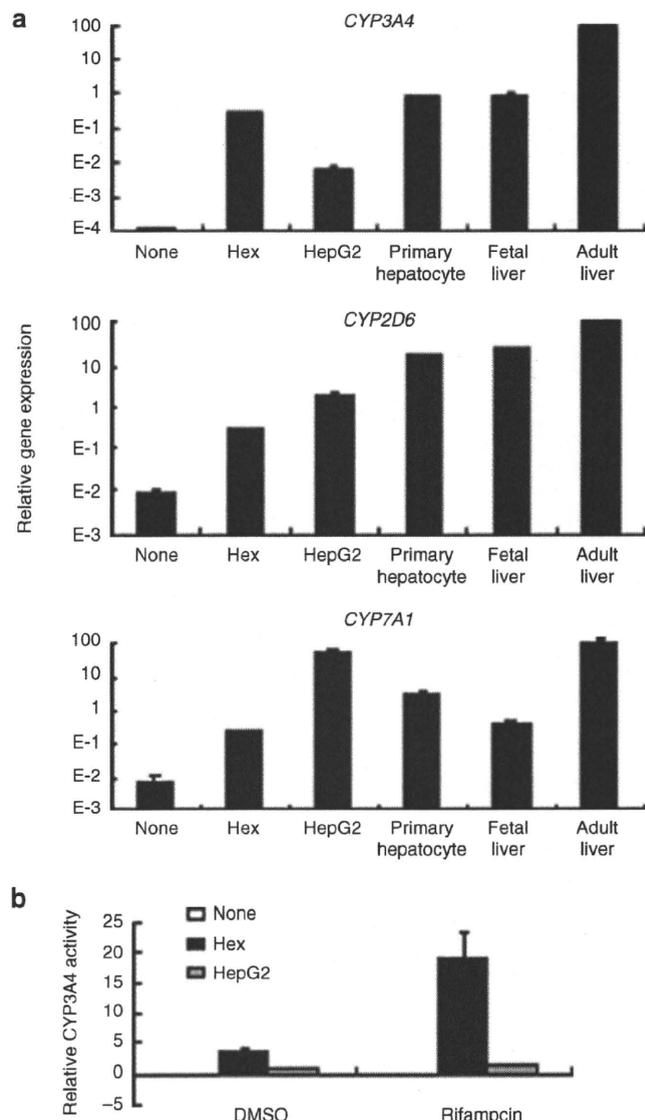


**Figure 5** Efficient differentiation of Ad-HEX-transduced hepatoblasts into hepatocytes. **(a,b)** Real-time RT-PCR analysis of **(a)** AFP and **(b)** ALB expression in nontransduced cells and Ad-HEX-transduced cells, both of which were induced from the human iPSC (Tic) derived definitive endoderm (day 0, 5, 6, and 12). The cells were transduced with Ad-HEX at day 6 as described in **Figure 1a**. The data at day 6 were obtained before the transduction with Ad-HEX. The graphs represent the relative gene expression level when the level in the fetal liver was taken as 100. **(c–j)** Immunocytochemistry of ALB, CYP3A4, CYP7A1, and CK18 expression in **(c–f)** nontransduced cells and **(g–j)** Ad-HEX-transduced cells, all of which were induced from the human iPSC (Tic) derived definitive endoderm at day 18. Nuclei were stained with DAPI. Bar = 50 μm. Ad, adenovirus; AFP, α-fetoprotein; ALB, albumin; CK18, cytokeratin 18; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; None, nontransduced cells; RT-PCR, reverse transcriptase-PCR.

definitive endoderm, and the Ad-HEX-transduced cells could differentiate into both hepatocytes and cholangiocytes.

**Directed hepatic differentiation from hepatoblasts**

With the protocol described above, heterogeneous populations containing CK7-positive cholangiocytes were observed at day 12 (**Figure 4p**). To promote the differentiation of hepatoblasts to hepatocytes, the human iPSC-derived differentiated cells at day 9 (**Supplementary Figure S5e**) were dislodged with trypsin-EDTA and plated on collagen I-coated dishes as previously reported.<sup>11</sup> After 8–11 days in culture with medium containing FGF4, HGF, OSM, and DEX, the Ad-HEX-transduced cells became more flattened (**Supplementary Figure S5m**), whereas the nontransduced cells became fibroblast-like cells (**Supplementary Figure S5i**). Gene expression analysis showed the upregulation of ALB mRNA in Ad-HEX-transduced cells under this culture condition, whereas the expression of ALB mRNA was reduced in the nontransduced cells at day 18 (**Figure 5b**). Immunostaining showed that only a small percentage of Ad-HEX-transduced cells expressed ALB at day 12 (**Figure 4p**), whereas most of the Ad-HEX-transduced cells were ALB-positive at day 18 (**Figure 5g**). Most of the Ad-HEX-transduced cells also expressed CYP3A4 at day 18 (**Figure 5h**). More importantly, in the Ad-HEX-transduced cells, CYP7A1 and cytokeratin 18 were detected and these proteins are known



**Figure 6** Cytochrome P450 isozymes in human iPSC (Tic) derived hepatocytes. **(a)** Real-time RT-PCR analysis of CYP3A4, CYP7A1, and CYP2D6 expression in iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, and fetal and adult liver tissues. **(b)** Induction of CYP3A4 by rifampicin in human iPSC (Tic) derived nontransduced cells, Ad-HEX-transduced cells, the HepG2 cell line and primary human hepatocytes, which were cultured 48 hours after plating the cells. Data are presented as the mean ± SD from triplicate experiments. The graphs represent the relative gene expression level when the level in the adult liver was taken as 100. AFP, α-fetoprotein; ALB, albumin; DMSO, dimethyl sulfoxide; ESC, embryonic stem cells; HEX, Ad-HEX-transduced cells; iPSC, induced pluripotent stem cell; LacZ, Ad-LacZ-transduced cells; None, nontransduced cells.

to be detected in hepatocytes but not in extra-embryonic cells<sup>31,32</sup> (**Figure 5i,j**). Quantitative analysis showed that ~84, 80, 88, and 92% of Ad-HEX-transduced cells expressed ALB, CYP3A4, CYP7A1, and cytokeratin 18, respectively. These results indicate that Ad-HEX-transduced cells could differentiate to hepatic cells. However, the expression level of ALB mRNA in Ad-HEX-transduced cells was lower than that in fetal liver tissue and in turn, the expression of AFP mRNA was maintained (**Figure 5a**). Therefore, Ad-HEX-transduced cells are committed to the hepatic lineage, but are not yet mature hepatocytes.

### Ad-HEX-transduced cells exhibit hepatic functions

To test the hepatic function in the Ad-HEX-transduced cells, we investigated the liver metabolism, because P450 cytochrome enzymes play a critical role in this function. We examined the expression level of several members of this multigene family, *i.e.*, *CYP3A4*, *CYP7A1*, mRNA and *CYP2D6* in Ad-HEX-transduced cells by real-time RT-PCR. The real-time RT-PCR analysis showed that the mRNAs for *CYP3A4*, *CYP7A1*, and *CYP2D6* were expressed in Ad-HEX-transduced cells, whereas none of these mRNAs were expressed in the nontransduced cells (Figure 6a). The expression levels of *CYP3A4* in Ad-HEX-transduced cells were similar to those observed in primary human hepatocytes, which were cultured 48 hours after plating the cells, or fetal liver tissues but lower than those in adult liver. The *CYP2D6* and *CYP7A1* mRNA expressions in Ad-HEX-transduced cells were lower than those in primary hepatocytes or adult tissues. Next, we investigated the metabolism of the P450 3A4 substrates by measuring the activity of P450 isozymes. The metabolites were detected in Ad-HEX-transduced cells, and their activity was 3.4-fold higher than that in the most commonly used human hepatocyte cell line, HepG2 (Figure 6b; DMSO column). This result was consistent with the real-time RT-PCR data (Figure 6a). We further tested the induction of *CYP3A4* upon chemical stimulation, because *CYP3A4* is the most prevalent P450 isozyme in the liver and is involved in the metabolism of a significant proportion of the currently available commercial drugs. Because *CYP3A4* can be induced with rifampicin, both Ad-HEX-transduced cells and HepG2 cells were treated with rifampicin, followed by treatment with *CYP3A4* substrate. Ad-HEX-transduced cells produced 5.4-fold higher levels of metabolites in response to rifampicin treatment (Figure 6b; rifampicin column). This result indicates that P450 isozymes are active in Ad-HEX-transduced cells.

### DISCUSSION

The object of this study was to develop an efficient method for generating hepatoblasts and hepatocytes from human ESCs and iPSCs for application to drug toxicity screening tests as well as therapeutics such as regenerative medicine. We found that transient HEX transduction in the definitive endoderm together with a culture under chemically defined conditions was useful for this purpose.

It has been reported that a high concentration of Activin A induces differentiation of human ESCs into the definitive endoderm.<sup>8,33,34</sup> On the other hand, undifferentiated human ESCs are maintained by a low concentration of Activin A.<sup>35</sup> Several studies have shown that bFGF promotes the differentiation of ESCs into the definitive endoderm and inhibits the differentiation of ESCs into the extra-embryonic endoderm.<sup>35–38</sup> bFGF has been reported to inhibit the BMP signaling, which can promote the extra-embryonic lineage differentiation.<sup>39</sup> The extra-embryonic endoderm expresses most of the hepatocyte markers, such as AFP.<sup>40</sup> Contamination of the extra-embryonic endoderm makes it difficult to estimate the hepatic differentiation from human ESCs and iPSCs.<sup>11,14,40</sup> In this study, we showed that both Activin A and bFGF induce definitive endoderm populations, while they repress the extra-embryonic endoderm differentiation (Figure 2g,h). Interestingly, after the differentiated cells that were cultured on

laminin-coated plates with Activin A and bFGF were passaged at day 5, FOXA2-positive cells (definitive endoderm) were enriched in the resultant cells at day 6 (Figure 2a–f). This may have been because FOXA2-positive cells efficiently adhered to the laminin-coated plate and/or because trypsinized, single undifferentiated ESCs/iPSCs cannot survive. The passaging of differentiated cells might be attributed to the reduction in the number of not only the extra-embryonic endoderm cells but also the undifferentiated cells. However, the efficiency of the definitive endoderm differentiation in this study was not as efficient as that reported by other groups.<sup>8,33,34</sup> Other cell lineages, such as the mesoderm and extra-embryonic endoderm, might remain at day 6 (Figure 2g,h and **Supplementary Figure S1**). Further improvement of the culture conditions will thus be needed in order to enhance the definitive endoderm differentiation.

Hepatoblasts and hepatocytes were differentiated from the human ESC- and iPSC-derived definitive endoderms by transient overexpression of the homeobox gene *HEX*. A fiber-modified Ad vector containing K7 peptides mediated much higher gene expression than conventional Ad vectors in the human ESC- and iPSC-derived definitive endoderms (**Supplementary Figure S6**). This new hepatic differentiation protocol shows that *HEX* induces AFP-positive hepatoblasts at day 9 and ALB-positive hepatocytes at day 12 from human ESCs and iPSCs, whereas the previous protocols require a few weeks or months to induce AFP- and ALB-positive hepatocytes from PSCs.<sup>9–11</sup> Previous studies suggested that *HEX* could regulate liver-enriched transcription factors such as hepatocyte nuclear factor 4A and hepatocyte nuclear factor 6.<sup>19,23</sup> Overexpression of the *HEX* gene under the conditions employed in the present study could activate several transcription factors that are required for hepatic differentiation (**Supplementary Figure S4a,b**). However, the Ad-HEX-transduced cells showed a low level of expression of *ALB* and some CYP450 species, as well as a high level of *AFP* expression, indicating that the cells were still immature. To promote further hepatic differentiation or maturation, it may be effective to culture the hepatic cells in a 3D environment or on feeder cells such as cardiomyocyte- or endothelium-derived cells.<sup>41,42</sup> In addition, the function of our hepatic cells was still limited. Further analysis of the other functions of our hepatic cells, such as glycogen storage, uptake of indocyanine green and organic anion low-density lipoprotein, and transplantation of Ad-HEX-transduced cells into the liver of immunodeficient mice, is clearly needed for the appreciation to drug screening and therapeutic treatment modalities.

During the preparation of this article, Kubo *et al.* have reported that *HEX* could promote hepatoblast differentiation from mouse ESCs.<sup>43</sup> Their report is consistent with our data, suggesting that *HEX* plays a pivotal regulatory role in not only mouse but also human hepatic differentiation. They also showed that the overexpression of *HEX* at the definitive endoderm stage is critical for hepatic specification of the mouse ESCs. We also confirmed that forced expression of *HEX* in the undifferentiated human ESCs and iPSCs did not elevate the expression of *ALB* and *CK7* (**Supplementary Figure S7**), indicating that *HEX* enhances the hepatic differentiation not from the undifferentiated cells but from the definitive endoderm. However, Kubo *et al.* used recombinant mouse ESCs (tet-*HEX* ESCs), in which the tetracycline-regulated *HEX* expression cassette