iPS 細胞を長期にわたって安定的に培養する方法をほぼ確立するにいたった。

D. 考察

ヒト iPS 細胞の未分化マーカーによる評価系をほぼ確立できた。そこでこの評価系を用いて培養下における経時的安定性、凍結融解後の細胞評価へ展開していくことが可能になったといえる。さらに創薬開発において非臨床データをヒトへの外挿性向上を図るためには従来のフィーダー細胞を用いた幹細胞培養条件では、再現性・安定性に問題が残る。そこで無フィーダ無血清培養条件で長期安定的に未分化性を維持できる細胞培養条件が課題となる。今回無フィーダー無血清培養下で長期にわたって安定した培養が可能となる条件をほぼ確立できた。今後さらに今回確立した未分化マーカーによる評価を加えるとともに、ゲノム安定性評価法を開発し品質管理・評価法の確立へとつなげていく。

E. 結論

ヒト iPS 細胞の未分化性安定評価法に関してヒト ES 細胞を基準とした定量的 PCR による評価法を確立した。今後は培養過程における未分化マーカーの遺伝子発現解析により経時的な評価を行い、iPS 細胞の細胞特性が維持できているかを検証する。さらに培養や凍結融解、継代による影響を評価するとともに、ゲノムレベルでの安定性に関しての解析を加えてることで品質評価技術の基盤整備を行っていく。また in vitro における多分化能安定性に関する評価法についても検討する。

F.健康危険情報

なし

G.倫理面への配慮

当研究所においては、ヒト間葉系細胞の樹立と基礎研究応用に関し、既に倫理審査を受け、承認を受けている（国立成育医療センター研究所、受付番号 25、26 及び 27、平成 15 年 1 月承認、受付番号 49、平成 15 年 10 月承認、受付番号 55、平成 15 年 11 月承認、受付番号 88、89、90、91 平成 16 年 7 月承認、受付番号 55、平成 16 年 11 月追加承認、受付番号 146、平成 17 年 4 月承認、受付番号 156、平成 17 年 7 月承認）。また、それぞれの組織については倫理的な手続きおよび考え方が年次毎に異なると予想され、「ヒト幹細胞等を用いる臨床研究に関する指針」に従い、最新の社会的な影響を十分に考慮する。なお、研究協力者に倫理専門家を加え、本研究遂行にあたって新たな倫理的問題が生じないよう、常にモニタリングを行い、必要に応じて意見交換を行う。

実験動物を用いる研究については、国立成育医療センター研究所動物実験指針に準拠して研究を実施する(承認番号 2003-002,2005-003)。特に、動物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめる。またその際、麻酔等手段により苦痛を与えない等の倫理的配慮をおこなう。実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行う。

H. 研究発表

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I. 知的財産権の出願・登録状況

1) 特許取得
なし

2) 実用新案登録
なし

3) その他
なし

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

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Seko Y, Azuma N, Takahashi Y, Makino H, Morito T, Muneta T, Matsumoto K, Saito H, Sekiya I, Umezawa A.	Human sclera maintains common characteristics with cartilage throughout evolution.	*PLoS ONE*	3	3709	2008
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Efficient osteoblast differentiation from mouse bone marrow stromal cells with polylysin-modified adenovirus vectors

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ABSTRACT

Bone marrow stromal cells (BMSCs) are expected to be a source for tissue regeneration because they can differentiate into multiple cell types. Establishment of efficient gene transfer systems for BMSCs is essential for their application to regenerative medicine. In this study, we compared the transduction efficiency in mouse primary BMSCs by using fiber-modified adenovirus (Ad) vectors, and demonstrated that AdK7, which harbors a polylysin (K7) peptide in the C-terminus of the fiber knob, could efficiently express a transgene in BMSCs. Notably, AdK7 robustly drove transgene expression in more than 90% of the BMSCs at 3,000 vector particles/cell. Furthermore, we showed that *in vitro* and *in vivo* osteogenic potential of BMSCs was dramatically promoted by the transduction of Runx2 gene using AdK7. These results indicate that this transduction system could be a powerful tool for therapeutic applications based on BMSCs.

Because bone marrow stromal cells (BMSCs) containing mesenchymal stem cells (MSCs) can be easily isolated from adult tissues and efficiently expanded *in vitro*, and can differentiate into multiple cell types [1,2], BMSCs are expected to be an ideal source of cells for the regeneration of tissues. However, it is difficult to obtain a large amount of pure differentiated cells from BMSCs because of their low differentiation efficiency. The cell transition from stem cells to lineage-committed cells involves many transcription factors that promote or suppress cellular differentiation [3]. Thus, to develop an efficient method for differentiating from BMSCs into specialized cells, we planned to combine the transduction of a functional gene, which promotes cellular differentiation, with stimulation by chemical reagents. To do this procedure, it is essential to develop efficient transduction systems for BMSCs.

Among the various types of gene delivery vectors, adenovirus (Ad) vectors have been widely used for gene transfer studies, since they can achieve high transduction efficiency and transduce both dividing and non-dividing cells [4]. Although Ad vector-mediated transduction into BMSCs has been performed, the transduction efficiency was found to be lower than those of many other cell lines [5,6]. This is due to the low levels of coxsackievirus and adenovirus receptor (CAR), which mediates adenovirus entry, on the cell surface [5,6]. To overcome this problem, we and others have generated several types of fiber-modified Ad vectors, which mediate efficient gene transduction into the cells expressing very low levels of CAR [7,8]. Transduction efficiency was improved in various types of the cells by the insertion of Arg-Gly-Asp (RGD) peptide or 7-tandem lysine residues (KKKKKKK: K7) peptide, which targets αv integrins or heparan sulfates, respectively, on the cell surface, into the fiber knob of the Ad vector [7,8]. In particular, we previously reported that polylysin-modified Ad vector (AdK7) is the most suitable vector for transduction into human bone marrow-derived MSCs (hMSCs) [9].

In this study, we initially investigated the transduction efficiency of mouse primary BMSCs by using fiber-modified Ad vectors. We next examined whether the osteogenic potential of BMSCs was promoted by using Ad vector-mediated transduction of a runt-related transcription factor 2 (Runx2) gene, which is known as a master gene for osteoblastogenesis [10,11].

Materials and methods

Ad vectors. Ad vectors were constructed using an improved *in vitro* ligation method [12,13]. The CA (cytomegalovirus (CMV) enhancer/β-actin promoter) promoter [14]-driven β-galactosidase

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(LacZ)-expressing plasmid, pHMCA-LacZ [15], was digested with I-*Ceu*I/PI-*Sce*I and inserted into I-*Ceu*I/PI-*Sce*I-digested pAdHM15-RGD [16] or pAdHM41-K7 (C) [8], resulting in pAdRGD-CA-LacZ, pAdK7-CA-LacZ, respectively. The CMV or the human elongation factor (EF)-1α promoter-driven LacZ-expressing plasmid, pHMCMV-LacZ [15] or pHMEF-LacZ [15], respectively, was also digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested-pAdHM41-K7 (C), resulting in pAdK7-CMV-LacZ or pAdK7-EF-LacZ, respectively. The CA promoter-driven mouse Runx2-expressing plasmid, pHMCA-Runx2, was generated by inserting a mouse Runx2 cDNA, which is derived from pCMV-Runx2 (a kind gift from Dr. S. Takeda, Tokyo Medical and Dental University, Tokyo, Japan) [17], into pHMCA5. pHMCA-Runx2 was also digested with I-*Ceu*I/PI-*Sce*I, and inserted with pAdHM4 [12] or pAdHM41-K7 (C), resulting in pAd-CA-Runx2 or pAdK7-CA-Runx2, respectively. Ad vectors (Ad-CA-LacZ, AdRGD-CA-LacZ, AdK7-CA-LacZ, AdK7-CMV-LacZ, AdK7-EF-LacZ, Ad-CA-Runx2, and AdK7-CA-Runx2) were generated and purified as described previously [18]. Determination of virus particle (VP) and biological titer were determined using by a spectrophotometrical method [19] and by means of an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA), respectively. The ratio of the biological-to-particle titer was 1:14 for Ad-CA-LacZ, 1:35 for AdRGD-CA-LacZ, 1:42 for AdK7-CA-LacZ, 1:25 for AdK7-CMV-LacZ, 1:32 for AdK7-EF-LacZ, 1:17 for Ad-CA-Runx2, and 1:28 for AdK7-CA-Runx2.

Mouse primary BMSCs. Primary BMSCs were harvested from female C57BL/6 mice (8 weeks; Nippon SLC, Shizuoka, Japan) as below. Femora and tibias were isolated and placed in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO)/20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin. Bone marrow was obtained by flushing these bones, and cells recovered from the bones of one animal were then seeded into a 150 mm tissue culture plate. Medium was changed every 2 days to remove non-adherent cells, and adherent cells were cultured until reaching confluence. At confluence, BMSCs were passaged after digestion with 0.25% trypsin/1 mM EDTA. BMSCs (passage 4–12) were subsequently used for further analysis.

LacZ assay. BMSCs (1×10^4 cells) were plated in 24-well plates. The next day, they were transduced with the indicated doses of Ad vectors for 1.5 hr. Two days later, X-gal staining and β-gal luminescence assays were performed as described previously [18].

Osteoblasts differentiation. BMSCs (1×10^4 cells) were plated in 24-well plates. Cells were transduced with 3000 VP/cell of Ad vector for 1.5 hr. After aspirating the viral solution, osteogenic differentiation medium, consisting of growth medium (DMEM/20% FBS) containing 50 μg/mL ascorbic acid 2-phosphate (Sigma), 5 mM β-glycerophosphate (Sigma), and 100 nM dexamethasone (Wako, Osaka, Japan), was added. The medium was replaced every 3 days.

von Kossa staining, calcium quantitation. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) and stained with $AgNO_3$ by the von Kossa method. To measure calcium deposition, cells were washed twice with PBS and decalcified with 0.5 M acetic acid, and cell culture plates were rotated overnight at room temperature (R/T). Insoluble material was removed by centrifugation. The supernatants were then assayed for calcium with the calcium C-test Wako kit (Wako). DNA in pellets was extracted using the DNeasy tissue kit (Qiagen), and calcium content was then normalized to DNA.

ALP assay. Cells were lysed in 10 mM Tris–HCl (pH 7.5) containing 1 mM $MgCl_2$ and 0.1% Triton X-100, and the lysates were then used for assay. Alkaline phosphatase (ALP) activity was measured using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. The protein concentration of the lysates was determined using a Bio-Rad assay kit (Bio-Rad laboratories, Hercules, CA), and ALP activity was then normalized by protein concentration.

RT-PCR. RT-PCR was performed as described previously [18]. The sequences of primers were as follows: Runx2(F), 5′-CCT CTG ACT TCT GCC TCT GG-3′; Runx2(R), 5′-CAG CGT CAA CAC CAT CAT TC-3′; osterix(F), 5′- CTT AAC CCA GCT CCC TAC CC-3′; osterix(R), 5′- TGT GAA TGG GCT TCT TCC TC-3′; bone sialoprotein(F), 5′- AAA GTG AAG GAA AGC GAC GA-3′; bone sialoprotein(R), 5′-GTT CCT TCT GCA CCT GCT TC-3′; osteocalcin(F), 5′-GCG CTC TGT CTC TCT GAC CT -3′; osteocalcin(R), 5′-TTT GTA GGC GGT CTT CAA GC-3′; collagen Iα1(F), 5′-CAC CCT CAA GAG CCT GAG TC-3′; collagen Iα1 (R), 5′-GCT ACG CTG TTC TTG CAG TG-3′, GAPDH(F), 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH(R), 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Western blotting. Western blotting was performed as described previously [18]. Briefly, lysates (20 μg) were subjected to 12.5% polyacrylamide gel and were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with Immunoblock (DS Pharma Biomedical, Osaka, Japan) at R/T for 1 hr, the membrane was exposed to rabbit anti-Runx2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight, followed by horseradish peroxidase-conjugated secondary antibody at R/T for 1 hr. The band was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ) and the signals were read using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). All blots were stripped and reblotted with antibody against β-actin (Sigma) for normalization.

In vivo heterotopic bone formation. BMSCs (2×10^6 cells, passage 8–9) were transduced with AdK7-CA-LacZ, AdK7-CA-Runx2, or Ad-CA-Runx2, at 3000 VP/cells for 1.5 hr. The next day, cells were collected by trypsin, and resuspended in 150 μl of PBS, and then injected into the hind limb biceps muscle of nude mice (Nippon SLC) (2animal/ group). At 4–5 weeks after injection, mice were anesthetized by isofluorane and bone formation was analyzed with a microcomputed tomography (microCT) system (eXplore Locus CT System; GE Healthcare, London, ON, Canada). Both an X-ray image and a three-dimensional reconstitution image were obtained by using the microCT system.

Results

Optimization of transduction efficiency in BMSCs by using various types of Ad vectors

To optimize Ad vectors for transduction into BMSCs, we prepared three LacZ-expressing Ad vectors, Ad-CA-LacZ, AdRGD-CA-LacZ, and AdK7-CA-LacZ. We investigated the transduction efficiency of these Ad vectors in BMSCs at the indicated vector dose. X-gal staining showed that LacZ-positive cells were less than 10% even at a dose of 3000 vector particles (VP)/cell in Ad-CA-LacZ (Fig. 1A). On the other hand, more than 90% of the cells expressed LacZ at the same dose in AdK7-CA-LacZ. A luminescence assay revealed that, at 3000 VP/cell, the LacZ expression level in the cells transduced with AdRGD-CA-LacZ or AdK7-CA-LacZ was increased by about 5- or 50-fold, respectively, in comparison with that in the cells transduced with Ad-CA-LacZ (Fig. 1B). These results were quite similar to those of our previous report, in which efficient transduction in hMSCs was achieved by using AdK7 [9], and our data clearly demonstrated that AdK7 is a suitable vector for transduction into both mouse BMSCs and hMSCs.

We and others reported that the choice of promoters is important for transduction efficiency, especially in immature cells [15,18,20,21]. Thus, we examined the transduction efficiency by comparing the promoter activities in BMSCs. In addition to the CA promoter, we prepared LacZ-expressing AdK7 under the control of the CMV promoter or the EF-1α promoter (AdK7-CMV-LacZ or AdK7-EF-LacZ, respectively). A luminescent assay showed that the CA promoter represented the highest transgene expression

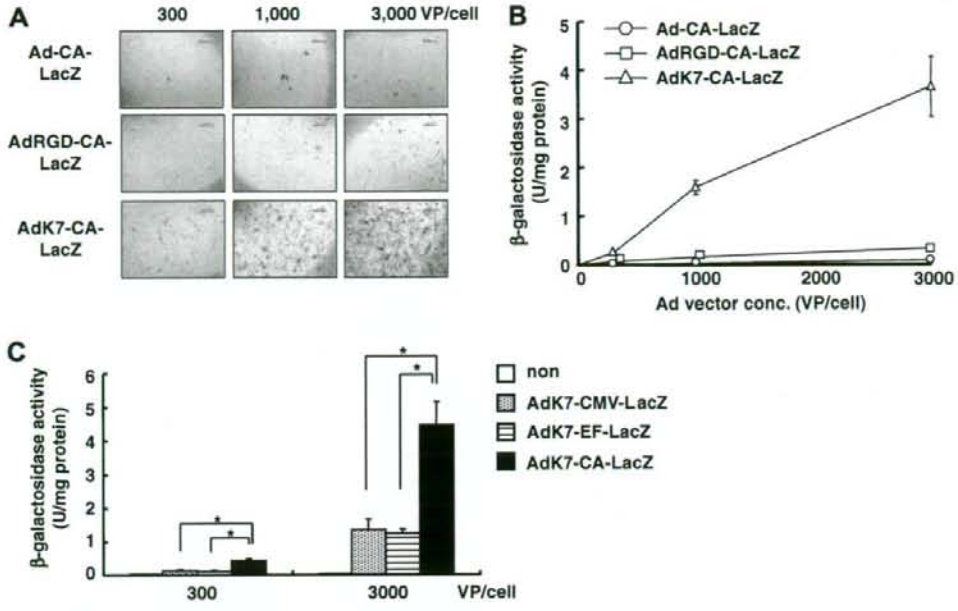

Fig. 1. Gene transduction efficiency in mouse primary BMSCs by various types of Ad vectors. Mouse BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors. Two days later, (A) X-gal staining and (B) luminescence assay were performed. Similar results of X-gal staining were obtained in three independent experiments. Scale bar indicates 200 μm. (C) Optimization of promoter activity in BMSCs using LacZ-expressing AdK7. BMSCs were transduced with the indicated dose of each Ad vector, and LacZ expression in the cells was measured. The data are expressed as mean ± S.D. ($n = 3$). $p < 0.01$.

among the three types of the promoters (Fig. 1C). These results demonstrate that AdK7 containing the CA promoter is the most effective at attaining high transduction efficiency in mouse BMSCs.

We also investigated the cytotoxicity in BMSCs transduced with AdK7-CA-LacZ. Almost no difference in cell number between non-transduced cells and AdK7-CA-LacZ-transduced cells was observed on day 2 after transduction (data not shown), indicating that AdK7 is an excellent vector with high transduction activity and low cytotoxicity in BMSCs.

Efficient osteoblast differentiation in vitro and in vivo by fiber-modified Ad vectors

Because an efficient method for transduction into BMSCs could be established by using AdK7 containing the CA promoter, we expected that efficient differentiation into specialized cells from BMSCs might be achieved by using this Ad vector. To test this, we generated mouse Runx2-expressing Ad vectors, AdK7-CA-Runx2 and Ad-CA-Runx2, because a Runx2 gene is both necessary and sufficient for mesenchymal cell differentiation towards osteoblast lineage [3]. Western blot analysis showed that Runx2 protein levels in AdK7-CA-Runx2-transduced cells were quite higher than those in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells (Fig. 2).

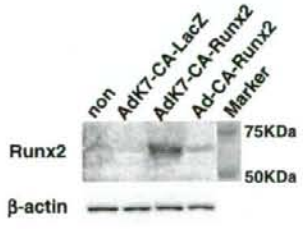

Fig. 2. Runx2 expression in Ad vector-transduced BMSCs. Cell lysates were isolated from BMSCs 2 days after the transduction, and Western blotting was performed.

We next assessed osteoblast differentiation by measuring alkaline phosphatase (ALP) activity, which is a marker of early osteoblast differentiation. After transduction with Ad vector, BMSCs were cultured in osteogenic differentiation medium for the indicated number of days. As shown in Fig. 3A, the ALP activity levels in AdK7-CA-Runx2-transduced cells were extremely increased in comparison with control cells. Notably, AdK7-CA-Runx2 mediated approximately 50-fold higher ALP activity than non-transduction or AdK7-CA-LacZ on day 5 after transduction. These results indicated that early osteoblast differentiation of BMSC was facilitated by AdK7-CA-Runx2. Because mature osteoblasts are known to be specialized in the production of extracellular matrix and the mineralization [22], we next examined the matrix mineralization in BMSCs. von Kossa staining revealed that matrix mineralization in AdK7-CA-Runx2-transduced cells was dramatically increased in comparison with non-, AdK7-CA-LacZ, or Ad-CA-Runx2-transduced cells (Fig. 3B, left). Furthermore, we observed a significant elevation of calcium deposition in AdK7-CA-Runx2-transduced cells even on day 5 after transduction, while neither non-transduced cells nor AdK7-CA-LacZ-transduced cells showed mineralization until day 15 (Fig. 3B, right). Ad-CA-Runx2 mediated slightly higher levels of calcium deposition than non-transduced or AdK7-CA-LacZ-transduced cells, but significantly lower levels than AdK7-CA-Runx2-transduced cells. Additionally, we found that the expression levels of marker genes characteristic of osteoblast differentiation, such as Runx2, osterix, bone sialoprotein, osteocalcin, and type I collagen, were also increased in AdK7-CA-Runx2-transduced cells (Fig. 3C). These results demonstrated that a conventional method using only osteogenic differentiation medium is

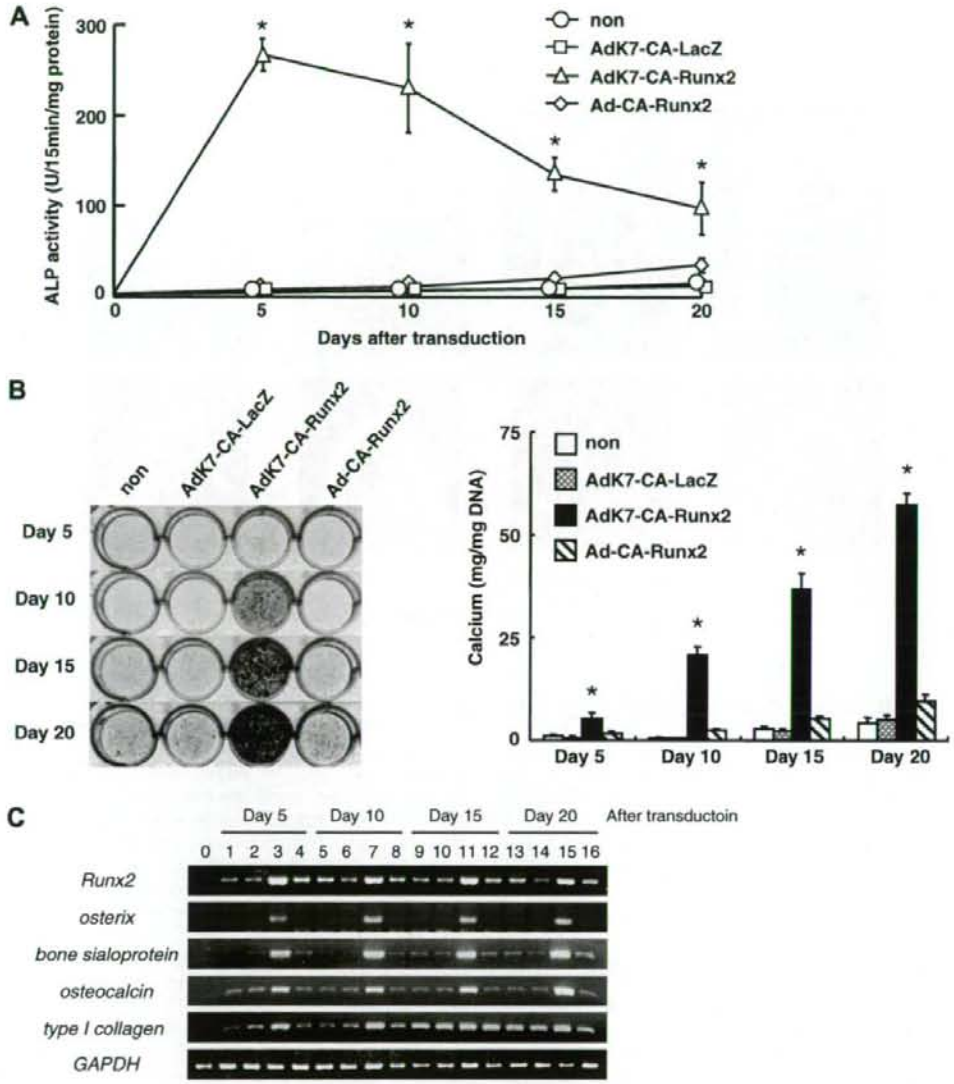

Fig. 3. Promotion of *in vitro* osteoblastic differentiation in AdK7-CA-Runx2-transduced BMSC. After transduction with each Ad vector at 3000 VP/cell for 1.5 hr, BMSCs were cultured for the indicated number of days. (A) ALP activity, (B, left) matrix mineralization, and (B, right) calcium deposition in the cells was determined. The data are expressed as mean ± S.D. ($n = 3$). $p < 0.01$ as compared with non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells. (C) RT-PCR was performed 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.

not enough for efficient osteoblast differentiation, and that, by efficient Runx2 transduction using AdK7, osteoblastogenesis of BMSCs could be dramatically accelerated *in vitro*.

Finally, to examine whether the increased levels of Runx2 expression in BMSCs could enhance the osteogenic potential of BMSC *in vivo*, BMSCs transduced with each Ad vector were injected into the hind limb biceps muscle of nude mice. Microcomputed tomography analysis revealed that no bone formation was observed in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells, while new bone was detected in mice injected with AdK7-CA-Runx2-transduced cells (Fig. 4), indicating that AdK7-CA-Runx2-transduced BMSCs efficiently differentiated into mature osteoblasts *in vivo*. These results clearly showed that AdK7-CA-Runx2 could facilitate the osteogenic potential of BMSCs both *in vitro* and *in vivo*.

Discussion

Because genetic manipulation is considered to be a powerful tool to promote cellular differentiation, it is necessary to establish efficient methods for transduction into BMSCs. Many researchers have reported that transduction efficiency of rat or human MSC was increased by using fiber-modified Ad vectors, such as AdRGD or Ad vectors containing Ad35 fiber knob and

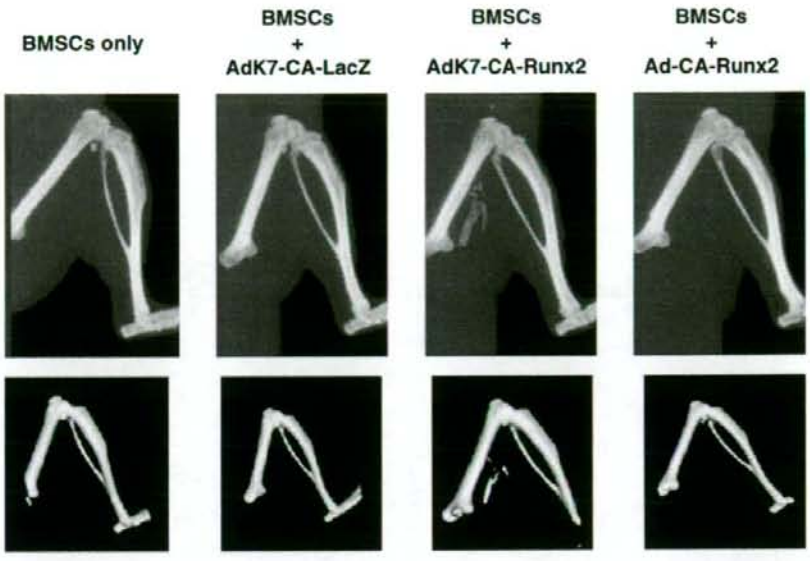

Fig. 4. *In vivo* ectopic bone formation of mouse BMSCs by AdK7-mediated Runx2 gene transduction. BMSCs were transduced with indicated Ad vectors at 3000 VP/cell. On the following day, cells were injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by the microCT system. Similar results were obtained in two independent experiments. Upper: X-ray images; lower: 3D reconstitution images.

shaft (AdF35) [23–25]. In this study, we demonstrated that AdK7 could express a transgene in BMSCs more efficiently than conventional Ad vector or AdRGD (Fig. 1A and B). Similarly, we have previously shown that the highest transduction efficiency in hMSC could be achieved by using AdK7, but not AdRGD or AdF35 [9]. Therefore, our data indicate that AdK7 is the most appropriate vector for various mesenchymal cells. We also found that the CA promoter showed higher gene expression in BMSCs than did the CMV or EF-1α promoter (Fig. 1C). This appears to be due to the potent activity of the CA promoter in immature cells [18,20]. Hence, we conclude that AdK7 containing the CA promoter is the most suitable vector for transduction into BMSCs.

We demonstrated that osteoblastogenesis of BMSCs was dramatically promoted by using AdK7-mediated Runx2 transduction (Figs. 3 and 4). This is the first study to report the usefulness of AdK7 in the field of stem cell differentiation. Runx2 is known to regulate osteoblastogenesis by controlling the expression of multiple osteoblast marker genes [10]. Because Runx2 protein and mRNA were highly expressed for more than 20 days in AdK7-CA-Runx2-trasduced cells (Figs. 2 and 3C), the expression of marker genes and ALP activity would be increased and would thereby enhance both *in vitro* and *in vivo* osteogenic ability. On the other hand, osteoblast differentiation could not be facilitated by AdK7-CA-Runx2 when osteogenic supplements were removed (data not shown), suggesting that osteogenic supplements were required for matrix mineralization, although differentiation efficiency was low when using only osteogenic supplements. Thus, efficient osteoblast differentiation of BMSCs would be achieved by the synergistic effect of both osteogenic supplements and efficient Runx2 transduction.

Unlike the case with AdK7-CA-Runx2, almost no osteoblast differentiation was seen in Ad-CA-Runx2-transduced cells. However, several groups reported that the osteogenic potential of MSCs was enhanced by Runx2 transduction using the conventional Ad vectors [26,27]. This difference would be attributable to the difference in transduction efficiency in BMSCs using the conventional Ad vector, because they showed that approximately 30–40% of the cells expressed transgenes by conventional Ad vector at 250–500 infectious units (ifu)/cell. Although we could not obtain high transduction efficiency using the conventional Ad vector, we showed that more than 90% of the cells were transduced by using AdK7-CA-LacZ at only 71 ifu/cell (3000 VP/cell) (Fig. 1A), without any decrease in viability (data not shown). Our results indicate that vector doses can be reduced by using AdK7, leading to a decrease in cytotoxicity to the cells. Therefore, AdK7, but not other fiber-modified Ad vectors or conventional Ad vectors, would contribute to safe regenerative medicine procedures.

In summary, we succeeded in developing efficient methods both for transducing mouse BMSCs and differentiating osteoblasts from BMSCs. Recently, many researchers have reported that mesenchymal stem/stromal cells could be isolated from adipose or placental tissues [28,29]. Because these mesenchymal cells are shown to possess mostly the same properties as BMSCs, AdK7 could probably be applied to these cells. Thus, our transduction methods can be a valuable tool for therapeutic applications based on adult mesenchymal stem/stromal cells.

Acknowledgments

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RESEARCH ARTICLE

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 β-galactosidase-expressing Ad vectors under the control of four different promoters (cytomegalovirus (CMV), rouse sarcoma virus, human elongation factor-1α, and CMV enhancer/β-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 β-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 (PPARγ) gene, which has been shown to be indispensable for adipogenesis [13,14], or a CCAAT/enhancer binding protein alpha (C/EBPα) 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 PPARγ1 gene, which is derived from pHMCMV6-PPARγ1 (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-PPARγ1. pHMCA5-PPARγ2 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-PPARγ1, because murine PPARγ2 cDNA encodes an additional thirty amino acids N-terminal to the first ATG of murine PPARγ1 [20]. Murine C/EBPα cDNA, which is derived from pEF-C/EBPα (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/EBPα. pHMCA5-PPARγ1, pHMCA-PPARγ2, or pHMCA5-C/EBPα 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-PPARγ1, pAdHM4-CA-PPARγ2, or pAdHM4-CA-C/EBPα, 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/β-actin promoter (CA) promoter, or the human elongation factor-1α (EF-1α) 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-PPARγ1, 1 : 8 for Ad-CA-PPARγ2, and 1 : 9 for Ad-CA-C/EBPα.

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 1×10^5 cells/ml, and 3×10^3 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 β-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-PPARγ1, Ad-CA-PPARγ2 or Ad-CA-C/EBPα) 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 a Bio-Rad Protein 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

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-β-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/EBPα 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: PPARγ(F), 5′-CCCTGGCAAAGCATTTGTAT-3′; PPARγ(R), 5′-AATCCTTGGCCCTCTGAGAT-3′; C/EBPα(F), 5′-CGCTGGTGATCAAACAAGAG-3′; C/EBPα(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.

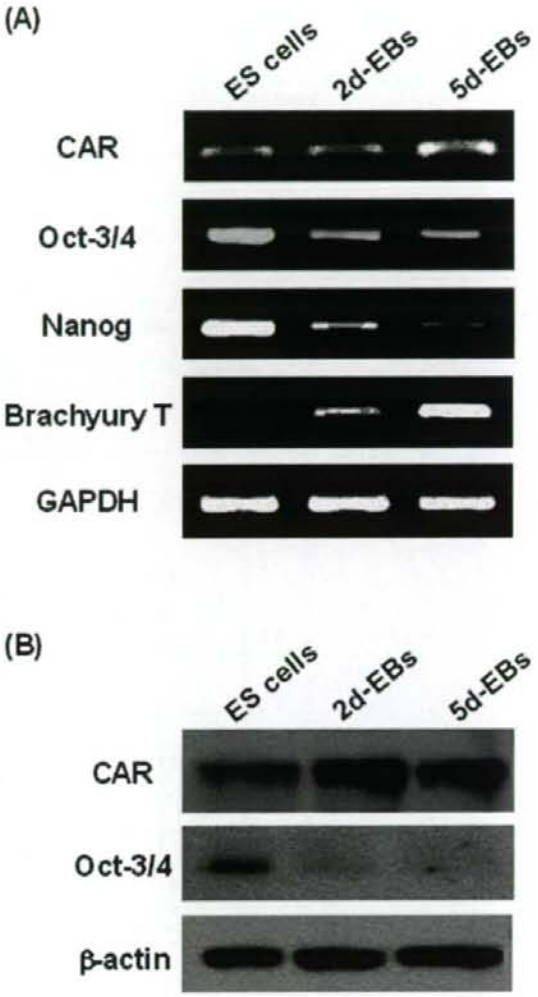

Figure 1. RT-PCR and Western blot analysis of ES cells and EBs. Total RNA or whole cell lysates were isolated from ES cells (lane 1), 2d-EBs (lane 2), or 5d-EBs (lane 3). RT-PCR (A) and Western blotting (B) were carried out as described in Materials and Methods. Abbreviations: ES cells, embryonic stem 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-1α 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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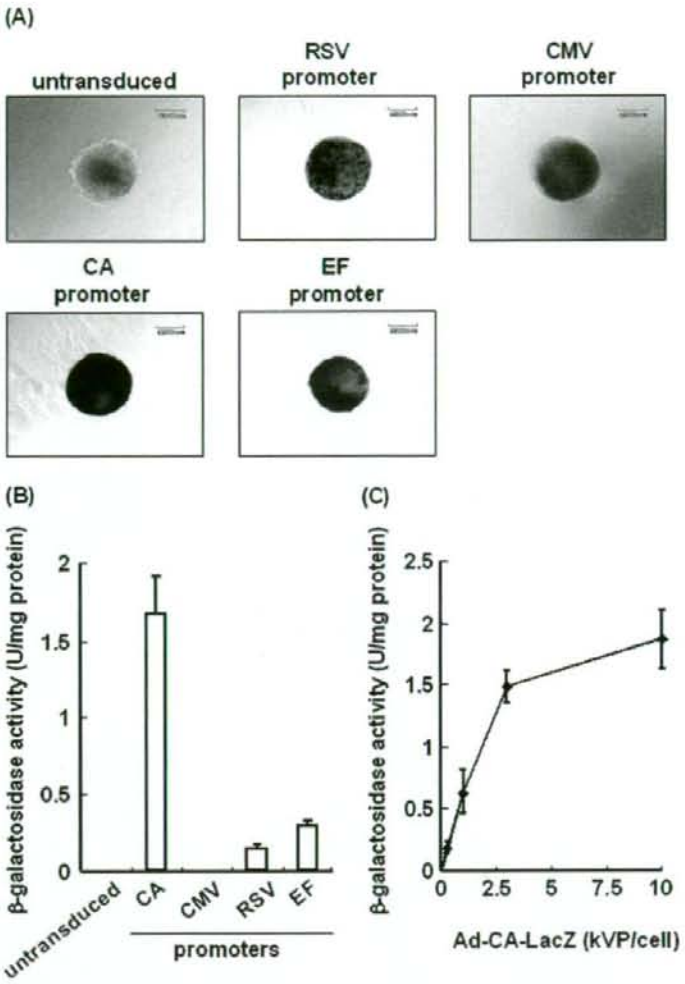

Figure 2. Ad vector-mediated transduction efficiency in EBs as determined using various types of promoters. 5d-EBs were transduced with Ad vectors at 3000 VP/cell for 2 days. After 48 h, X-gal staining (A) and β-galactosidase luminescence assay (B) were performed as described in Materials and Methods. 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

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.

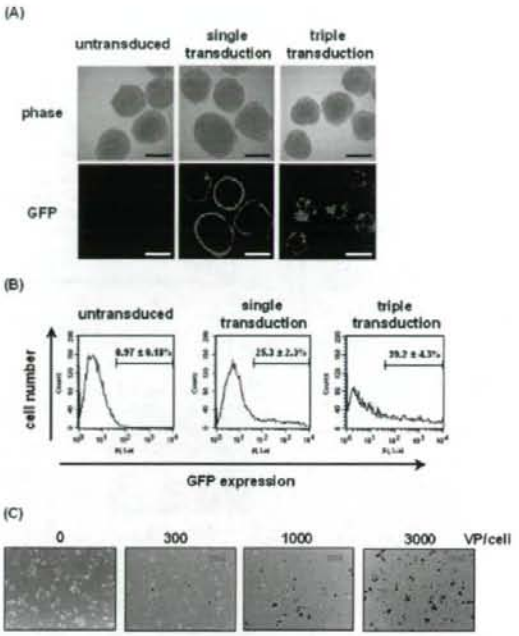

Figure 3. Optimization of gene transfer into EBs by Ad vector. EBs were transduced with 10 000 VP/cell of Ad-CA-GFP by single transduction (A, B; middle) or triple transduction (A, B; right). On day 7, (A) confocal microscopic analysis and (B) flow cytometric analysis were performed. The data are expressed as mean ± S.D. (n = 5). Untransduced EBs are represented as a negative control (A, B; left). Scale bar indicates 300 μm. (C) 5d-EB-derived single cells 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.

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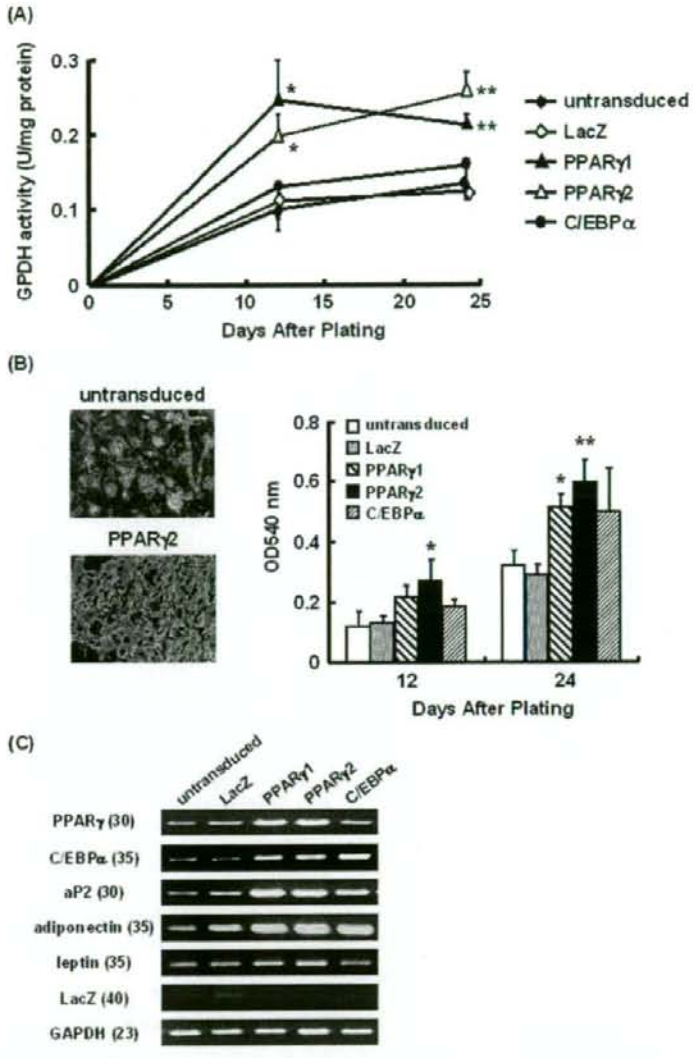

Figure 4. Efficient adipocyte differentiation from EBs by Ad vector-mediated PPARγ gene transfer. EBs were transduced in triplicate with 10 000 VP/cell of Ad-CA-LacZ, -PPARγ1, -PPARγ2, or -C/EBPα. After plating onto a gelatin-coated dish on day 7, EBs were cultured for 24 days with adipogenic supplements. 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

might be essential for adipocyte differentiation from ES cells, and this might be why trypsinized EBs showed lower differentiation. Therefore, EBs in the sphere form were then treated with each Ad vector by triple transduction, and GPDH activity was measured after cultivation with adipogenic supplements. The levels of GPDH activity in the cells transduced with Ad-CA-LacZ or Ad-CA-C/EBPα were similar to those of untransduced EBs. On the other hand, it was significantly increased by Ad vector-mediated PPARγ (PPARγ1 and PPARγ2) expression at days 12 and 24 (Figure 4A). Moreover, Oil-red O staining revealed that 70–80% of the cells transduced with PPARγ were Oil-red O positive, whereas 50–60% were LacZ- or C/EBPα-transduced cells or untransduced cells (data not shown). In particular, many large lipid droplets accumulated in the cells transduced with Ad-CA-PPARγ1 or Ad-CA-PPARγ2 compared to untransduced EBs (Figure 4B, data not shown). We also confirmed the expression of marker genes of adipocyte differentiation by semi-quantitative RT-PCR analysis. As shown in Figure 4C, PPARγ, C/EBPα, adipocyte-specific fatty acid binding protein (aP2), and adiponectin expression were up-regulated in PPARγ1- and PPARγ2-transduced EBs (Figure 4C). Furthermore, when we measured the expression levels of LacZ mRNA to examine whether Ad vector-mediated transduction still continued, its expression was undetectable in Ad-CA-LacZ-transduced EBs (Figure 4C), suggesting that expression of PAPRγ or C/EBPα, which was observed in PPARγ- or C/EBPα-transduced EBs, would not be derived from the Ad vector but from endogenous genes.

Next, we examined whether Ad vector-mediated transduction into EBs could increase the differentiation efficiency even in the absence of adipogenic supplements. Adipogenesis from EBs was promoted by transduction of PPARγ, although the levels of GPDH activity and lipid droplet accumulation could not achieve the adipogenic supplements-treated levels (Figure 5 and data not shown). These results indicate that Ad vector-mediated transduction of the PPARγ gene into EBs could improve the efficiency of adipocyte differentiation from ES cells with or without adipogenic supplements.

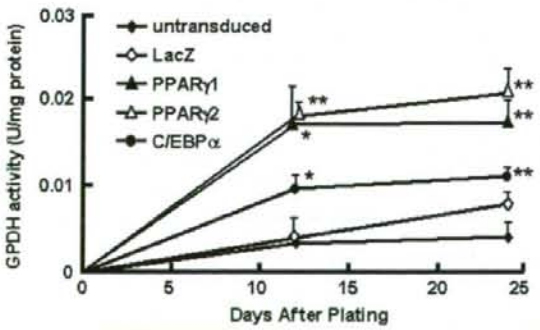

Figure 5. Ad vector-mediated transduction into EBs promotes adipogenesis in the absence of adipogenic supplements. EBs were transduced in triplicate with 10 000 VP/cell of each Ad vector, and then GPDH activity was measured after cultivation in differentiation medium without adipogenic supplements. The data are expressed as mean ± S.D. (n = 3). *$p < 0.05$ and **$p < 0.01$, respectively, as compared with untransduced EBs

Discussion

In the present study, we compared the transduction efficiency of four types of promoters (RSV, CMV, CA, and EF-1α), which are widely used in transduction experiments, in EBs by using Ad vector, and demonstrated that the CA promoter could robustly drive transgene expression in EBs (Figures 2A and 2B). We concluded that the CA promoter was the most appropriate promoter for transduction into EBs. We also showed that in trypsinized EBs, more than 90% of the cells were transduced with the Ad vector containing the CA promoter, and that a transgene could be successfully expressed in the interior of EBs by triple transduction (Figure 3). We and other groups have demonstrated that the CA promoter is potentially active in mES cells [11,32], human $CD34^+$ cells [33,34], and embryos of transgenic mice [35], suggesting that the CA promoter is active particularly in immature cells including stem cells. EBs are thought to be composed of immature cells because of the presence of Oct-3/4 and Nanog, although their expression levels are moderate (Figure 1). Thus, the CA promoter is useful in attaining high levels of transgene expression in EBs. Interestingly, the CMV promoter, which is one of the strongest promoters known so far, had little activity not only in mES cells [11,32], but also in EBs (Figures 2A and 2B). This might be due to the defense response against the transcription of foreign genes using a non-cellular promoter in immature cells. Rust *et al.* [36] reported that the CMV promoter was active in cardiac myocytes derived from mES cells in spite of being inactive in undifferentiated ES cells. These results suggest that the CMV promoter, in contrast to the CA promoter, does not work in both EBs and ES cells, and it is possible that transcriptional silencing might occur through some mechanism such as the DNA methylation of the CMV promoter [37]. However, Rufaihah *et al.* recently showed that about 90% of the human 7d-EB-derived single cells were transduced with an Ad vector containing the CMV promoter [38]. Although it is unknown why the CMV promoter has potent activity in human EBs, the transcriptional silencing using the CMV promoter might occur in murine but not human cells. Thus, the silencing mechanism in the CMV promoter in immature cells should be further investigated.

Differentiation procedures from ES cells by gene delivery have been performed using long-term constitutive expression systems such as those involving retrovirus vector [4,7]; however, these procedures might be not suitable for therapeutic use. Ad vectors could be useful because of their transient expression. However, few studies have been performed to differentiate ES cells into functional cells using transient expression systems. In the

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present study, we showed that transient PPARγ transduction into EBs by using an Ad vector could enhance adipocyte differentiation in the presence or absence of adipogenic supplements (Figures 4 and 5). Adipocyte differentiation, however, was not enough in the absence of adipogenic supplements compared with that in the presence of it (Figure 5), suggesting that it is important to combine PPARγ transduction and treatment with adipogenic supplements to attain efficient adipcyte differentiation. Analysis of semi-quantitative RT-PCR revealed that the LacZ mRNA expression level by the Ad vector was quite low in EBs at day 24, indicating that Ad vector-mediated transduction is sufficient to trigger differentiation into functional cells even though its expression is transient and could not be introduced into all the cells composed of EBs. These observations lead to the expectation that an Ad vector-mediated transient gene expression system could be applied to differentiate ES cells into other cells such as osteoblasts, hematopoietic cells, and so on.

We also showed that both PPARγ1 and PPARγ2 significantly promoted the adipocyte differentiation from EBs (Figures 4 and 5). Our results indicate that overexpression of PPARγ1 and PPARγ2 was capable of accelerating adipogenesis in EBs, and that the N-terminal domain of PPARγ2 is not necessarily required for its adipogenic activity in EBs since both have similar activities. These results are consistent with the previous report [13]. In the absence of adipogenic supplements, C/EBPα-transduced EBs also exhibited a higher GPDH activity than LacZ-transduced EBs or untransduced EBs (Figure 5), indicating that C/EBPα as well as PPARγ has the potential to promote adipogenesis from EBs. However, in the presence of adipogenic supplements, C/EBPα could not increase the efficiency of adipocyte differentiation (GPDH activity) as much as could PPARγ, despite the increased expression of aP2 and adioponectin mRNA and a slight accumulation of lipid droplets in the EBs (Figure 4). C/EBPα has been shown to play important roles in adipogenesis, but its role is limited to the induction and retention of PPARγ levels [16]. It is possible that because C/EBPα could not robustly elicit endogenous PPARγ expression in the adipogenic supplements treated condition (Figure 4C) or adipogenic supplements might conceal the effect of C/EBPα, the efficiency of adipocyte differentiation in EBs transduced with C/EBPα might be similar to untransduced EBs. These results suggest that transient gene delivery into EBs using an optimized Ad vector could not only facilitate the efficiency of differentiation into functional cells, but could also be useful for the analysis of gene functions.

In summary, we have shown that an Ad vector containing the CA promoter has superior transduction efficiency for EBs and that the Ad vector system has potential use in basic research, particularly that regarding stem cell differentiation. Thus, this system might be a valuable tool for the molecular switching of cellular differentiation and could be applied to regenerative medicine based on ES cells.

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