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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

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was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ, USA) and the signals were read using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). For the detection of internal control, a monoclonal anti-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

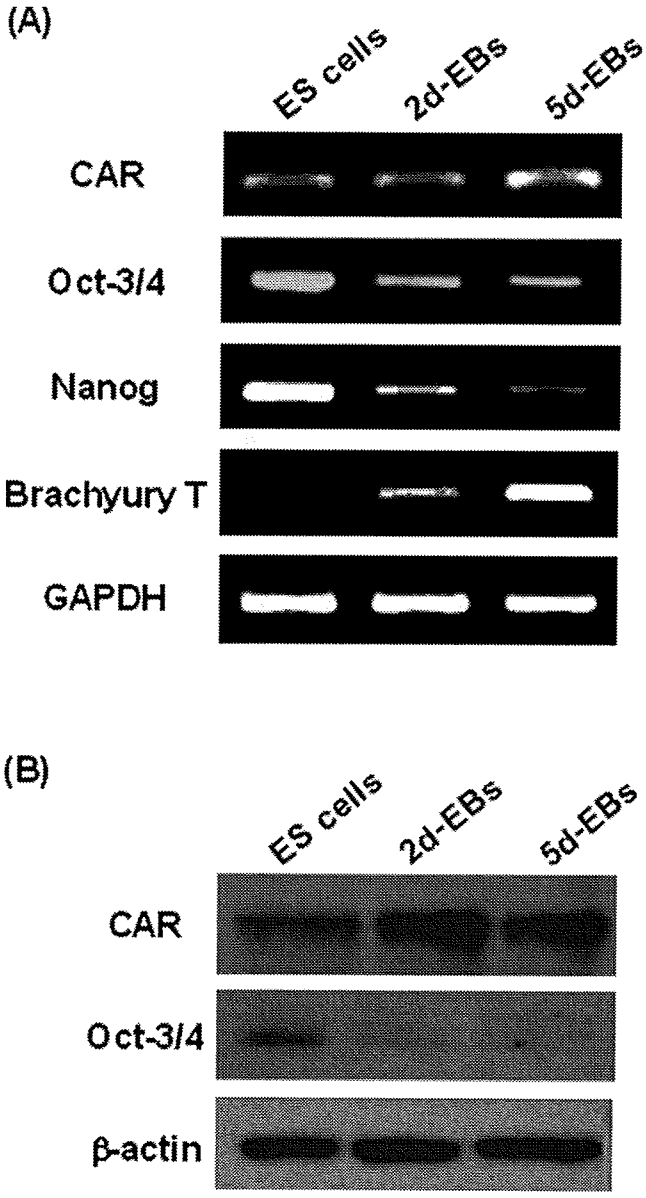

Figure 1. RT-PCR and Western blot analysis of ES cells and EBs. Total RNA or whole cell lysates were isolated from ES cells (lane 1), 2d-EBs (lane 2), or 5d-EBs (lane 3). RT-PCR (A) and Western blotting (B) were carried out as described in Materials and Methods. Abbreviations: ES cells, embryonic stem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

be introduced into EBs by using a conventional Ad vector.

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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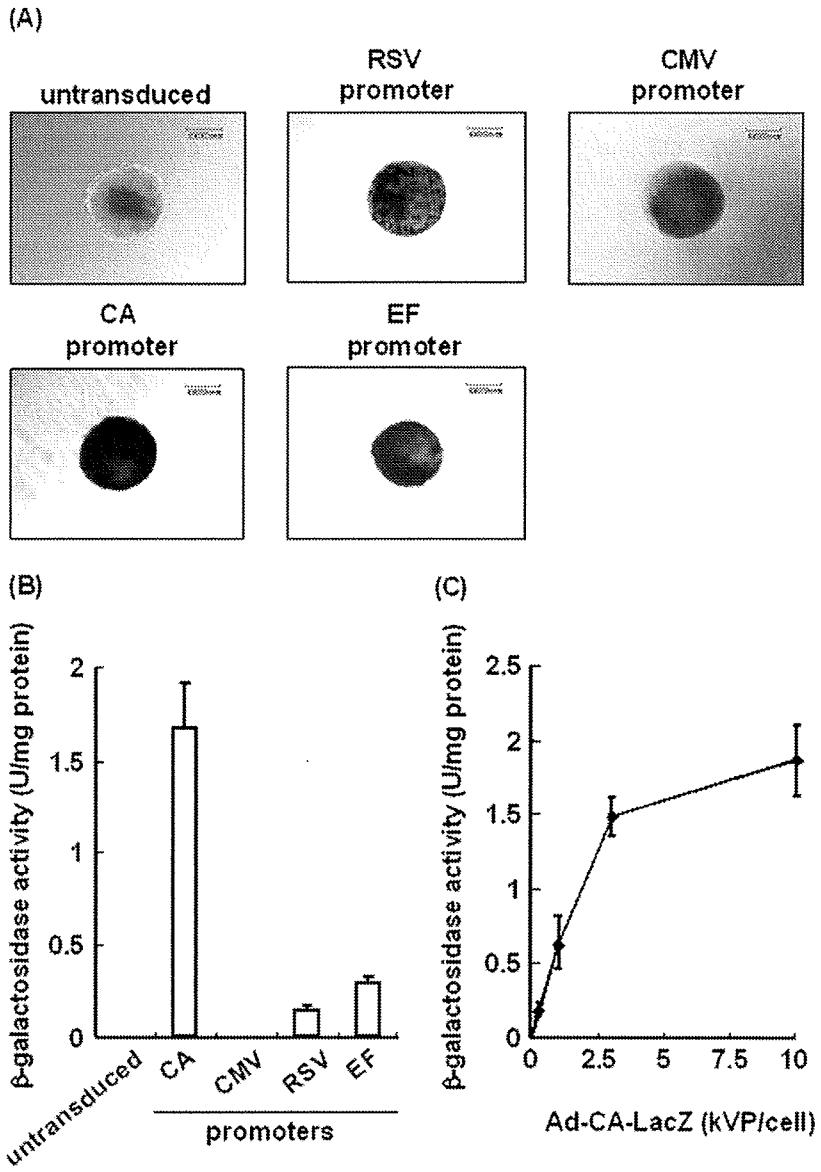

Figure 2. Ad vector-mediated transduction efficiency in EBs as determined using various types of promoters. 5d-EBs were transduced with Ad vectors at 3000 VP/cell for 2 days. After 48 h, X-gal staining (A) and β-galactosidase luminescence assay (B) were performed as described in Materials and Methods.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

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

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

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

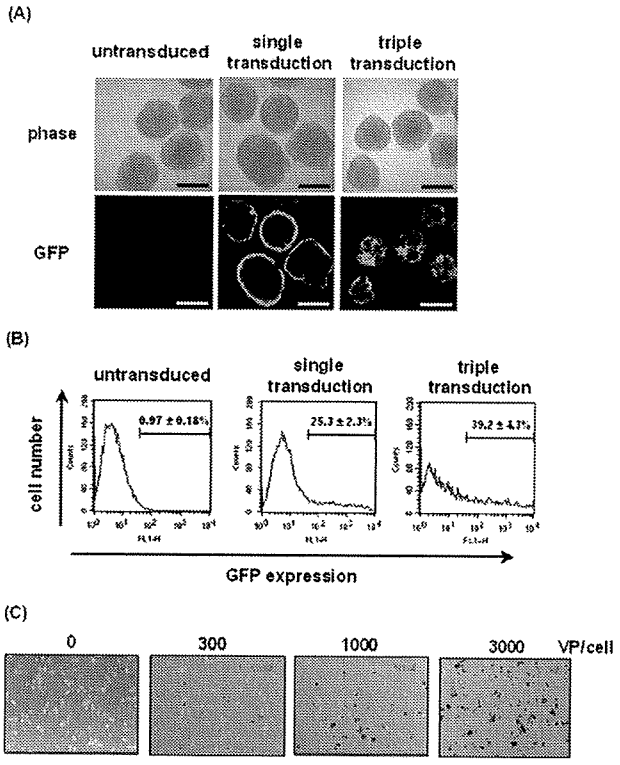

Figure 3. Optimization of gene transfer into EBs by Ad vector. EBs were transduced with 10 000 VP/cell of Ad-CA-GFP by single transduction (A, B; middle) or triple transduction (A, B; right). On day 7, (A) confocal microscopic analysis and (B) flow cytometric analysis were performed. The data are expressed 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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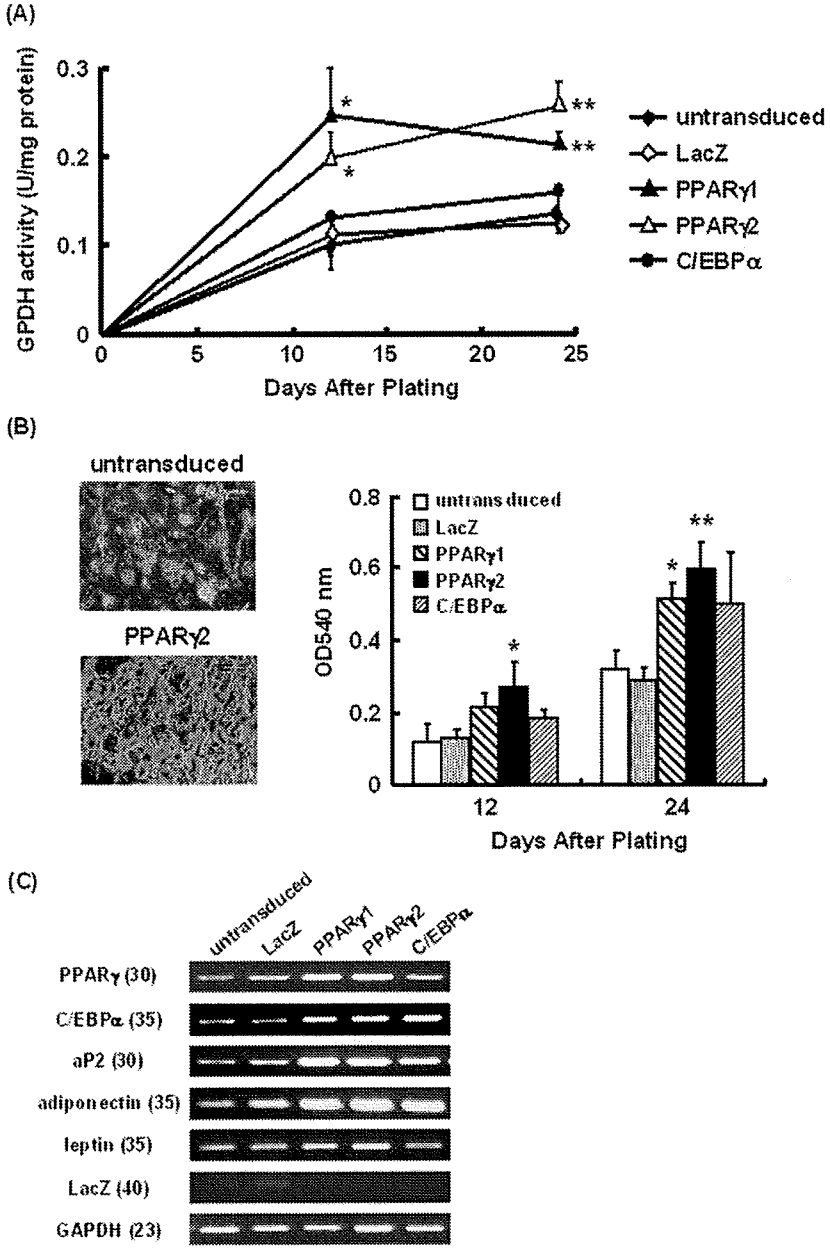

Figure 4. Efficient adipocyte differentiation from EBs by Ad vector-mediated PPARγ gene transfer. EBs were transduced in triplicate with 10 000 VP/cell of Ad-CA-LacZ, -PPARγ1, -PPARγ2, or -C/EBPα. After plating onto a gelatin-coated dish on day 7, EBs were cultured for 24 days with adipogenic supplements.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

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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.

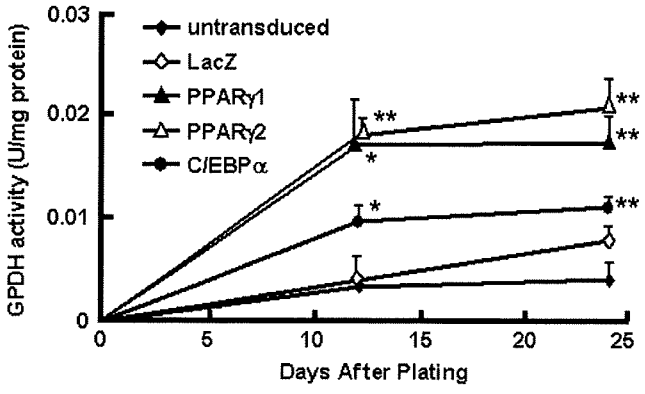

Figure 5. Ad vector-mediated transduction into EBs promotes adipogenesis in the absence of adipogenic supplements. EBs were transduced in triplicate with 10 000 VP/cell of each Ad vector, and then GPDH activity was measured after cultivation in differentiation medium without adipogenic supplements. The 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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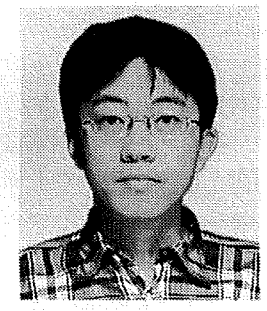
各種幹細胞への高効率遺伝子導入法の開発とその応用

川端健二　独立行政法人 医薬基盤研究所基盤的研究部遺伝子導入制御プロジェクト



1992年に京都大学薬学部を卒業後，同 大学院薬学研究科薬剤学教室(橋田 充教授)に進学し，プラスミドDNAを用いた遺伝子治療に関する研究に従事．1997年に薬学博士の学位を取得．学位取得後，大阪府立母子保健総合医療センター研究所免疫部門(長澤丘司部長，現 京都大学教授)に流動研究員および日本学術振興会特別研究員して所属し，ケモカインSDF-1(CXCL12)およびその受容体CXCR4遺伝子欠損マウスにおける造血機能の解析研究に従事．

2002年，国立医薬品食品衛生研究所遺伝子細胞医薬部研究員(山口照英部長)として採用される．2004年より同 研究所大阪支所医薬基盤研究施設に異動，2005年4月より独立行政法人医薬基盤研究所基盤的研究部遺伝子導入制御プロジェクト主任研究員(水口裕之プロジェクトリーダー)，現在に至る．

現在の主な研究テーマは，改良型アデノウイルスベクターを用いた各種幹細胞の高効率分化法の開発，およびアデノウイルスベクターによる自然免疫応答の分子メカニズムの解明．

(かわばた けんじ)

幹細胞(stem cells)は，自己複製能と分化多能性という大きく二つの特長を有する細胞であり，過去には概念としてその存在が唱えられていたが，幹細胞マーカーの発見や機能アッセイ法の確立により，現在では実体として捉えられるようになった．幹細胞と一口に言っても，造血幹細胞，神経幹細胞，間葉系幹細胞，ES細胞など多種の幹細胞が発見されている．また最近では，腫瘍組織の一部の細胞が幹細胞様の性質を有しており，この細胞の存在が放射線治療や化学療法を困難にしているというがん幹細胞(cancer stem cells)仮説も提唱されている．

これらの幹細胞は，種々の細胞に分化可能であることから治療への応用が考えられ，そのなかでも造血幹細胞は実際に骨髄移植療法のなかで汎用されている．また，1998年にはヒトES細胞が胚盤胞内部細胞塊から樹立されたことにより，幹細胞を再生医療へ応用することが一層期待されている．しかしながら，幹細胞を直接生体に移植するには困難な場合も多く，たとえばES細胞はマウス生体に投与するとランダムに分化しテラトーマ(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のち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，培養液に特定の液性因子を加える方法がとられているが分化効率は充分ではない．

そこで，筆者ら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出来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充分な導入効率が得られない．

アデノウイルスベクターは，CAR(coxsackievirus and adenovirus receptor)とよばれる膜蛋白質を受容体として感染し，高効率・一過性に外来遺伝子を発現させることが可能な系として遺伝子治療や基礎研究に汎用されているベクターであるが，多くの幹細胞のようにCARを発現していない細胞に対しては遺伝子導入することが出来ない．筆者の所属する研究室では，従来型(5型)アデノウイルスベクターだけでなく，CAR陰性の細胞に対しても高効率な遺伝子導入が可能となるように種々の改良型アデノウイルスベクターを開発してきた(図1)[1]．これらのベクターを用いることにより，ES細胞，間葉系幹細胞，造血幹細胞に対して，それぞ

若手研究者のひろば

Development of efficient gene delivery system into stem cells

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Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation

本欄はDDS研究に携わる若手研究者の自己紹介を兼ねて日頃の研究内容，研究成果を広くアピールする欄です．人選は本誌編集委員，およびDDS学会役員の推薦によります．

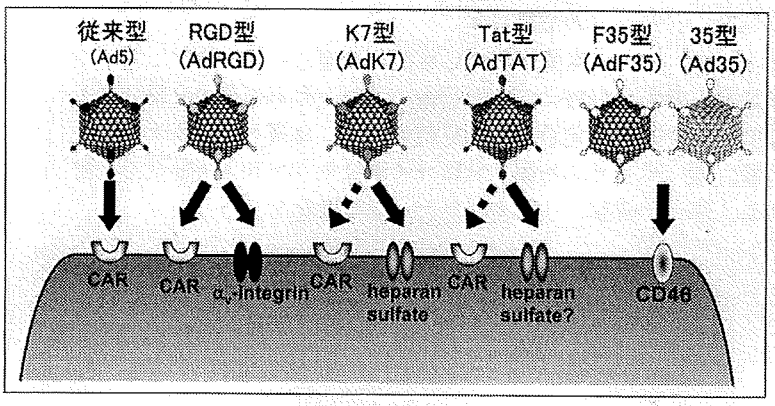

図1　筆者らが独自に開発した改良型アデノウイルスベクター

表1　改良型アデノウイルスベクターを用いた各種細胞への遺伝子導入効率

	従来型 Ad	改良型 Ad
ヒト造血幹細胞 (CD34陽性細胞)	5%以下	50%以上(Ad35)
ヒト間葉系幹細胞	10%以下	100%(AdK7)
マウスES細胞	10%以下	90%以上(Ad5-EF1α プロモーター)

れ従来型アデノウイルスベクターの10倍以上の遺伝子導入効率が達成可能となった(表1)[2]．また，最適化されたアデノウイルスベクターを用いて幹細胞の分化を制御することも試みている．マウスES細胞は，LIF(leukemia inhibitory factor)の存在下で未分化を維持できることが知られている．LIFはその受容体に結合後，下流のアダプター分子であるSTAT3(signal transducer and activator of transcription 3)を介して未分化シグナルを伝達する．

そこで，アデノウイルスベクターを用いてSTAT3のdominant-negative変異体(STAT3F)cDNAを導入しSTAT3シグナルを遮断したところ，LIF存在下であるにもかかわらず，ES細胞は中胚葉，外胚葉，内胚葉いずれにも分化することが明らかとなった．さらに，STAT3F cDNAとNanog(ES細胞の未分化維持に必須の転写因子)cDNAを共導入したところ，ES細胞は未分化を維持しつづけた．したがって，アデノウイルスベクターを用いて機能遺伝子を導入することによりES細胞の分化・未分化を自由に制御できる可能性が示された[3]．現在までに，骨，筋肉，血液など，各分化系列への分化に必須のマスター遺伝子がつぎつぎと明らかにされており，アデノウイルスベクターを用いてこれらの遺伝子を各種幹細胞に導入することにより，目的の細胞に効率よく分化誘導することが可能になるものと期待される．

幹細胞を目的の細胞に分化させたあとは，副作用軽減の観点から遺伝子発現はそれ以上持続しないことが望ましい．したがって，遺伝子導入による幹細胞の分化誘導にアデノウイルスベクター用いることの長所は，その高い遺伝子導入効率だけでなく，発現が一過性であることもあげられる．

これまで述べてきたように，アデノウイルスベクターは幹細胞を用いた再生医療への応用に重要なツールとなる可能性を有している．現在，「つぎはどんな細胞に分化させたら面白いか」ということを考えながら研究を進めているところである．

文　献

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改良型アデノウイルスベクターを用いた各種幹細胞への遺伝子デリバリー

特集　遺伝子・核酸医薬品のデリバリー

川端健二・櫻井文教[*1)]，水口裕之[*1,2)]

Gene delivery into stem cells by modified adenovirus vectors

The application of adenovirus (Ad) vectors, which are widely used in gene therapy, depends on CAR (coxsackievirus and adenovirus receptor) expression on the cells. To overcome this problem, the capsid proteins of Ad vectors have been genetically modified. Here, we introduce several types of capsid-modified Ad vectors. Furthermore, we describe the application of capsid-modified Ad vectors into some kinds of stem cells for regenerative medicine.

アデノウイルスベクターは，遺伝子治療や基礎研究に幅広く用いられている．しかしながら，アデノウイルスベクターの受容体であるCARの発現が乏しい細胞では，アデノウイルスベクターによる遺伝子導入効率は低い．そこで筆者らは，CAR非依存的に遺伝子導入可能な種々のカプシド改変型アデノウイルスベクターを開発してきた．本稿では，これらのカプシド改変型アデノウイルスベクターの特徴と，その応用例として，近年，再生医療分野で注目を浴びている各種幹細胞への高効率遺伝子導入法について解説する．

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key words : adenovirus vector, gene delivery, stem cells, regenerative medicine, gene therapy

ヒトアデノウイルスは，赤血球凝集活性の違いからAからFまでのサブグループにわけられ，計51種の血清型が存在する．遺伝子治療用ベクターとして繁用されているアデノウイルスベクターは，サブグループCに属するヒト5型アデノウイルスを基盤としている．5型アデノウイルスの感染は，カプシド蛋白質のファイバーが細胞表面に存在するCAR(coxsackievirus and adenovirus receptor)と結合することにより起こる．そのため，従来の5型アデノウイルスベクターは，CAR陽性細胞へは効率よく遺伝子導入可能であるが，CARの発現が乏しい細胞への遺伝子導入効率はきわめて低いことが課題であった．

CARの発現が乏しい細胞としては，造血幹細胞や間葉系幹細胞などの幹細胞，血液細胞，悪性度の高いがん細胞，血管内皮細胞などがあげられ，このような細胞へはアデノウイルスベクターの適用は不向きであった．

本稿では，CAR陰性細胞に対しても高効率遺伝子導入が可能な種々の改良型アデノウイルスベクターについて概説し，つぎにその応用例として，近年，再生医療への応用が期待されている幹細胞への高効率遺伝子導入法について紹介する．

改良型アデノウイルスベクター

1. ファイバー改変型アデノウイルスベクター

アデノウイルスのファイバーはノブ，シャフト，テール領域にわけられ，ノブ領域がCARと結合する(**図1a**)．アデノウイルスベクターの感染域を拡大するためのアプローチの一つとして，ファイバーノブのHIループやC末端領域に細胞接着活性などを有する外来ペプチドを遺伝子工学的に挿入することがあげられる．

筆者らは，これらの部位に外来ペプチドをコードした遺伝子をきわめて簡便に挿入できるファイバー

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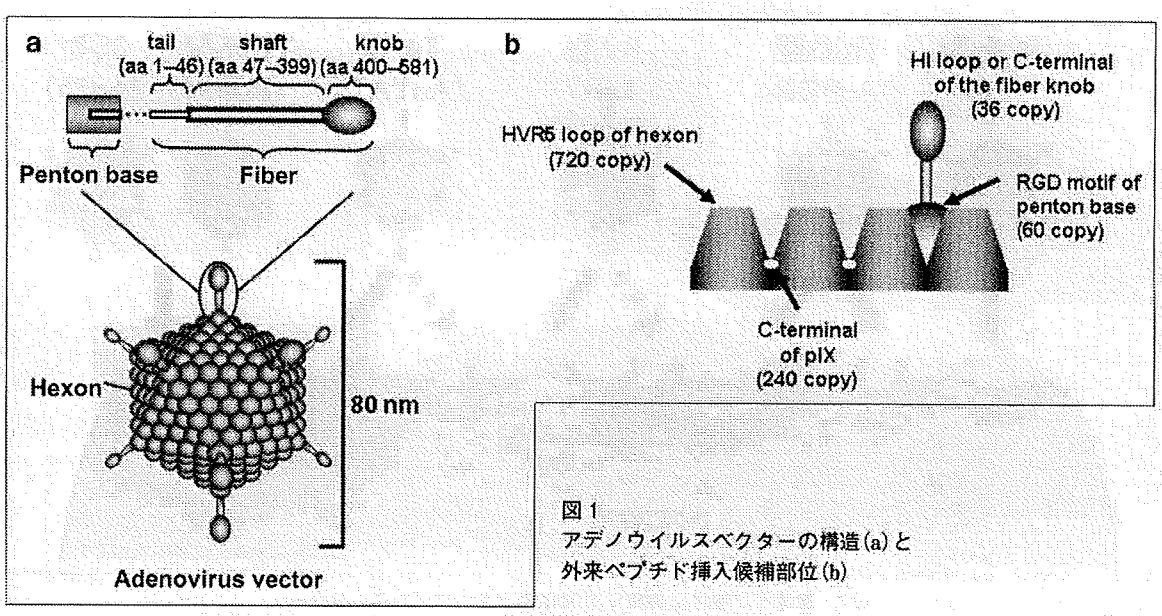

図1
アデノウイルスベクターの構造(a)と
外来ペプチド挿入候補部位(b)

改変型アデノウイルスベクター作製法を開発済みであり[1,2]，この技術と *in vitro* ライゲーションに基づいたE1欠損領域への外来遺伝子挿入法[3,4]を組み合わせることにより，CAR陰性細胞に対しても高効率に遺伝子導入が可能なアデノウイルスベクターを簡便に作製する方法を開発した(**図2**，**表1**)．ファイバーノブのHIループにRGD(リジン-グリシン-アスパラギン酸)からなるペプチドを挿入しインテグリンと親和性を保持させることにより(RGD型ベクター)，種々のがん細胞[5,6]や樹状細胞[7]，血管内皮細胞[8]に高効率な遺伝子導入が可能となった．また，ファイバーノブのC末端領域に七つのリジン残基からなるポリペプチドを挿入したファイバー改変型(K7型)アデノウイルスベクターでは，ヘパラン硫酸と親和性を有するようになり，種々のCAR陰性細胞に効率よく遺伝子導入が可能である[2,9]．

さらに筆者らは，最近，HIV(human immunodeficiency virus)由来のprotein transduction domain(PTD：蛋白質導入ドメイン)として知られているTatペプチド[10]をファイバーノブに付与することで，RGD配列やポリリジン配列を付与したベクターよりも，より広範に効率よく外来遺伝子を発現可能であることを見いだした．したがって，Tatペプチドを付与したアデノウイルスベクターは，遺伝子治療用ベクターや基礎研究におけるツールとしてきわめて有用であると考えられる．

2. ファイバー置換型アデノウイルスベクター

サブグループBに属する11型あるいは35型などのアデノウイルスはCARではなく，補体制御因子として知られているCD46を受容体として感染することが知られている[11,12]．

そこで筆者らは，5型アデノウイルスベクターのファイバー領域のみを35型アデノウイルスのものに置換したベクター(F35型ベクター)やすべての構造蛋白質を35型アデノウイルスからなるベクターを開発した(**図2**)[13~15]．ヒトにおいては，CD46は赤血球を除くほぼすべての細胞に発現していることが知られており，これらのベクターは，多くのヒト由来細胞だけではなく，たとえば5型アデノウイルスベクターでの遺伝子導入が困難で，遺伝子治療の重要な標的細胞であるCD34陽性ヒト造血幹細胞にも効率よく遺伝子導入可能であることが明らかとなった[14,16]．

3. ヘキソン，pIX改変型アデノウイルスベクター

ファイバーは，ウイルス1粒子当たり12分子存在するが(ファイバーは3量体を形成するため，ノブは36コピー存在する)，主要なカプシド蛋白質のヘキソンは240分子(同じく3量体をとっている

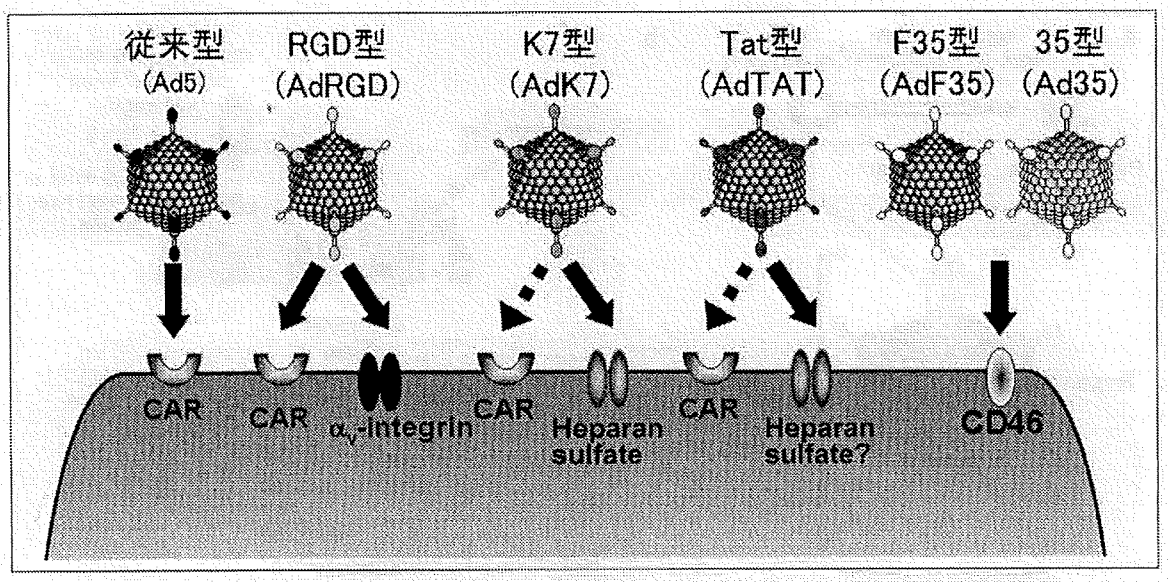

図2　筆者らが独自に開発した改良型アデノウイルスベクター

野生型のファイバーを持った従来の5型アデノウイルスベクターは細胞表面上の受容体であるCARを認識して感染するが，RGD配列やポリリジン配列をファイバーに有したファイバー改変型ベクターはCARだけでなく，αvインテグリンやヘパラン硫酸を認識しても感染できる．また，35型のアデノウイルスのファイバーを有したベクターや，すべての構造蛋白質が35型アデノウイルスからなるベクターは，CD46を認識して感染する．Tat型ベクターは未知の機構により(ヘパラン硫酸を介するという報告もある)細胞内に取り込まれる．

表1　改良型アデノウイルスベクターを用いた各種細胞への遺伝子導入効率

	従来型 Ad	改良型 Ad
ヒト造血幹細胞 (CD34陽性細胞)	5%以下	50%以上(Ad35)
ヒト間葉系幹細胞	10%以下	100%(AdK7)
マウスES細胞	10%以下	90%以上 (Ad5-EF1α-プロモーター)
樹状細胞 (ヒト・マウス)	10%以下	90%以上(AdRGD)
CAR陰性がん細胞 (ヒト・マウス)	10%以下	100%(AdRGD)
マウス脂肪細胞	10%以下	50%以上(AdK7)

ため，720コピー存在する)，pIX(プロテインIX)は240分子(240コピー)存在するため，これらの領域を改変できれば，より効率のよい遺伝子導入が可能になることが期待される(**図1b**)．ヘキソンは，ウイルス粒子の中で最も豊富に存在する蛋白質であり，カプシドの構造を維持する役割を有する．またpIXは，ヘキソンカプソマーの間に挟まれた形で存在し，ヘキソン同士の結合を補助する．

そこで，ヘキソンのhypervariable region 5 (HPV5)およびpIXのC末端に外来ペプチドを挿入できるベクター系を構築し，ファイバー改変型，ヘキソン改変型，pIX改変型各アデノウイルスベクターの遺伝子発現効率について比較検討した[17]．

各挿入部位にRGDペプチドを挿入した結果，ファイバーノブのHIループにRGDペプチドを挿入したファイバー改変型アデノウイルスベクターが最も高い遺伝子発現効率を示した．これは，ヘキソンやpIXと比較し，ファイバーノブは最も外側に位置するので，宿主細胞と結合しやすくなっていること，およびヘキソンやpIXに発現させたペプチドはファイバーによる立体障害のため，細胞表面に作用しにくくなっている可能性が原因として考えられる．

したがって，ヘキソンやpIXを改変する場合，ファイバーを遺伝子工学的に欠損させた(ファイバーレス)アデノウイルスベクターを基盤ベクターとすることにより遺伝子発現効率が改善する可能性が考えられ(この場合，同時にCAR経由による遺伝子導入も起こらないため，ターゲティングアデノウイルスベクターの開発にもつながる)，現在検討中である．

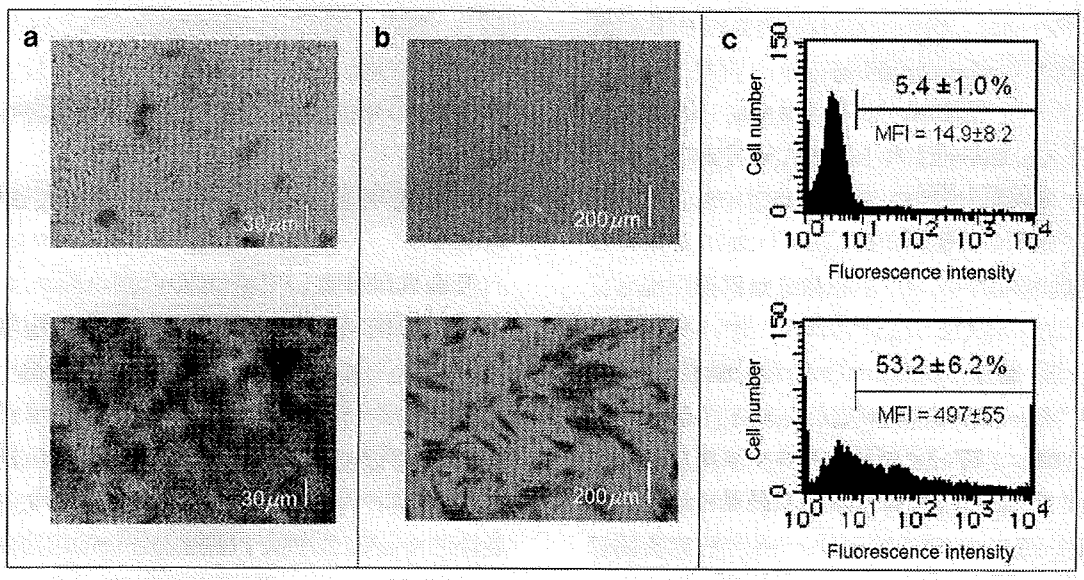

図3　改良型アデノウイルスベクターによる各種幹細胞への高効率遺伝子導入

a：マウスES細胞に対し，CMVプロモーター(上段)あるいはEF-1αプロモーター(下段)有するβ-ガラクトシダーゼ遺伝子を発現する従来型アデノウイルスベクターを感染させた．

b：ヒト間葉系幹細胞に対し，従来型(上段)あるいはK7型(下段)アデノウイルスベクターを用いてβ-ガラクトシダーゼ遺伝子を発現させた．

c：ヒト造血幹細胞に対し，従来型(上段)あるいは35型(下段)アデノウイルスベクターを用いてGFP遺伝子を発現させた．

ES細胞への高効率遺伝子導入法

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．近年，ヒトES細胞が樹立されたことにより，これを再生医療へ応用するための基礎研究が活発に行われている[18]．しかしながら，ES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，その原因の一つとしてES細胞への効率よい遺伝子導入法が確立されていないことがあげられる．

これまで，ES細胞に対しては，プラスミドDNAを用いたエレクトロポレーション法(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)[19]，レトロウイルスベクター[20]，レンチウイルスベクター[21]，ポリオーマウイルスの複製機構を利用したスーパート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細胞ではエピゾーマルに増幅できる性質を利用した方法)[22]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つづける方法であり，ES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．アデノウイルスベクターは，導入遺伝子が宿主染色体へ組み込まれることなく，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細胞を目的の機能細胞に分化させたあと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細胞に最も適したアデノウイルス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細胞はアデノウイルス受容体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[23]．また，RSV，CMV，CA(β-actin promoter/CMV enhancer)，EF-1αの4種のプロモーターを用いて検討した結果，ES細胞にはCAおよびEF-1α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(**図 3a**)．

これまでアデノウイルスベクターは，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ト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)である胚線維芽細胞にも遺伝子発現がみられたのに対し，EF-1α 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-1α プロモーターの活性が胚線維芽細胞にくらべ 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わけることによって，再生医療への幅広い応用が期待できる．

つぎに，最適化されたアデノウイルスベクターを用いて 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-1α プロモーターを有した従来型アデノウイルスベクターを用いて，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ころ，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．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，先述のベクターを用いて STAT3F と Nanog を同時に発現させたところ，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つづけた．したがって，アデノウイルス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[23]．現在，筆者らは ES 細胞に対し分化に関与するマスター遺伝子などを導入することにより，特定の細胞への分化制御が可能かどうか検討中である．

間葉系幹細胞への高効率遺伝子導入法

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出来る[24]．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一つとして，細胞分化に関与する遺伝子を導入することがあげられる．アデノウイルス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，ヒト間葉系幹細胞は CAR を発現していないためにその導入効率はきわめて低く，遺伝子導入には高タイターのベクターを必要としていた[25,26]．

筆者らは，種々のファイバー改変型アデノウイルス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．その結果，ヒト間葉系幹細胞には K7 型ベクターが最も適しており，従来型ベクターの 460 倍の遺伝子導入効率を示すことが明らかとなった(**図 3b**)[27]．RGD 型ベクターや F35 型ベクターは，従来型ベクターに比較しそれぞれ 16 倍，130 倍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．

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 型アデノウイルス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，さまざまな系列の細胞に分化するというだけではなく，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[28]．したがって，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など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.

造血幹細胞への高効率遺伝子導入法

造血幹細胞は成体では主に骨髄に存在し，すべての血液細胞に分化する能力を有する．造血幹細胞に対する遺伝子導入は，主にレトロウイルスベクターやレンチウイルスベクターが用いられてきたが，これらのベクターは半永久的に導入遺伝子を発現しつづけるため，治療目的に応用するには不都合が生じる場合がある．たとえば，MDR1(multi-drug resistance gene 1)遺伝子をレンチウイルスベクターを用いて造血幹細胞に導入した場合，導入遺伝子が細胞分化後も発現するため，生体(マウス)に移植した場合に白血病を発症するという報告がある[29]．また，重症複合性免疫不全症候群(X-SCID)の患者に対して行われたレトロウイルスベクターによる遺伝子治療では，ウイルスゲノムの染色体へのランダムな組込みにより白血病を発症するという副作用が起きた(がん遺伝子であるLMO2遺伝子の近傍に治療用遺伝子が挿入されたことが直接の原因である)[30]．したがって，再生医療への応用には，アデノウイルスベクターのように発現が一過性のベクターが好ましいことが多い．

しかしながら，ヒト造血幹細胞を含む画分であるCD34陽性細胞では，CAR陽性細胞は数%と少なく，5型アデノウイルスベクターによる遺伝子導入効率も4〜5%と低い．一方，ほぼすべてのCD34陽性細胞はCD46を発現しているため，35型アデノウイルスベクターを用いて遺伝子導入を行ったところ，50%以上の遺伝子導入効率が得られることが明らかとなった(**図3c**)[14]．また，CD34陽性細胞への遺伝子導入に適したプロモーターを探索した結果，EF-1α，CA，CMVi(イントロンAを付加したCMVプロモーター)などのプロモーターを用いることにより高い遺伝子発現が得られた[16]．

したがって，最適化されたアデノウイルスベクターを用いてHoxB4などの造血幹細胞の増殖に関与する遺伝子を導入することにより，これまで困難とされてきた造血幹細胞の*in vitro*での増幅が可能ではないかと考え，現在検討中である．また，*in vivo*においては細胞増殖や抗アポトーシス，ホーミングに関与する遺伝子を造血幹細胞に導入することにより，造血幹細胞を*in vivo*へ移植後の骨髄への生着細胞数が上昇する結果，移植効率の向上が得られる可能性が考えられ，これに関しても現在研究を進めている．

以上，各種幹細胞を用いた再生医療への応用において障害となっている遺伝子導入に関し，改良型アデノウイルスベクターの有用性について解説した．また**表1**には，これら幹細胞を含むさまざまな細胞種への改良型アデノウイルスベクターでの遺伝子導入効率の改善例について示した．今後，筆者らの開発したこれらのベクターが，幹細胞研究や再生医療研究などの基礎・応用研究に大きく貢献できることを期待している．

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"DDS" 用語解説 No. 112

ウイルスベクター (virus vector)

ウイルスベクターとは，ウイルス本来が持つ感染機構を利用して外来遺伝子を細胞に導入・発現させる遺伝子導入用ベクターである．ウイルスベクターでは，そのウイルスゲノムから自己複製に必須の遺伝子を取り除くことで自己複製不能となっており，外来遺伝子がウイルスゲノムに挿入されている．これまでにレトロウイルスベクター，アデノウイルス(Ad)ベクターをはじめ，多くのウイルスベクターが開発されており，2006年6月までの遺伝子治療臨床試験のうち，約70%でウイルスベクターが用いられている．これらウイルスベクターは，基本骨格となるウイルスの種類によりその遺伝子導入特性は大きく異なるが，プラスミドDNAを基本とした非ウイルスベクターと比較して，総じて遺伝子導入効率にすぐれる一方，ウイルスベクターの抗原性や感染域，作製方法の煩雑さ，ウイルスに対する抗体保持率などが問題となっている．

近年，これらの問題点を克服した次世代ウイルスベクターの開発研究が盛んに行われている．たとえば，subgroup Cに属する従来のAdベクターはAd受容体(CAR)を発現していない細胞(樹状細胞や血液細胞など)への遺伝子導入効率はきわめて低い．しかし，subgroup Bに属するAdベクター(35型Adベクターなど)はCD46を受容体として認識するため(ヒトCD46は赤血球を除くほぼすべての細胞に発現している)，ヒト造血幹細胞をはじめとする広範な細胞種に対し高効率な遺伝子導入が可能である．

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SHORT COMMUNICATION

Adenovirus serotype 35 vector-mediated transduction following direct administration into organs of nonhuman primates

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Adenovirus (Ad) serotype 35 (Ad35) vectors have attracted remarkable attention as alternatives to conventional Ad serotype 5 (Ad5) vectors. In a previous study, we showed that intravenously administered Ad35 vectors exhibited a safer profile than Ad5 vectors in cynomolgus monkeys, which ubiquitously express CD46, an Ad35 receptor, in a pattern similar to that in humans. However, the Ad35 vectors poorly transduced the organs. In this study, we examined the transduction properties of Ad35 vectors after local administration into organs of cynomolgus monkeys. The vectors transduced different types of cells depending on the organ. Hepatocytes and microglia were mainly transduced after the vectors were injected into the liver and cerebrum, respectively. Injection of the vectors into the femoral muscle resulted in the transduction of cells that appeared to be fibroblasts and/or macrophages. Conjunctival epithelial cells showed transgene expression following infusion into the vitreous body of the eyeball. Transgene expression was limited to areas around the injection points in most of the organs. In contrast, Ad35 vector-mediated transgene expression was not detected in any of the organs not injected with Ad35 vectors. These results suggest that Ad35 vectors are suitable for gene delivery by direct administration to organs.
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Keywords: *adenovirus serotype 35 vector; local administration; nonhuman primate; CD46*

Adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses with icosahedral symmetry. To date, 51 human adenovirus (Ad) serotypes have been identified and classified into six species.[1,2] Among these serotypes, Ad serotype 5 (Ad5), which belongs to species C, is the basis of almost all the Ad vectors commonly used, including those used in clinical trials. Conventional Ad5 vectors have several advantages as gene delivery vehicles. However, it is now well established that the hurdles to Ad5 vector-mediated gene therapy are the high seroprevalence to Ad5 in adults and the refractoriness of cells lacking the expression of coxsackievirus-adenovirus receptor, which is a primary receptor for Ad5, to Ad5 vectors. Pre-existing anti-Ad5 immunity significantly decreases the transduction efficiencies of Ad5 vectors. Even when an Ad5 vector-based vaccine was administered locally into muscle, pre-existing anti-Ad5 antibodies reduced its efficacy.[3,4] A lack of coxsackievirus-adenovirus receptor expression renders the cells unsusceptible to Ad5 vectors at least *in vitro*. Important target cells for gene therapy, including hematopoietic stem cells and dendritic cells, often poorly express coxsackievirus-adenovirus receptor. In addition to these drawbacks, Ad5 vectors have high hepatic tropism. Even when Ad5 vectors are locally injected into a diseased area (for example, a tumor), they are drained from the injection sites into the systemic circulation and primarily transduce hepatocytes because of their high hepatic tropism; on the other hand, efficient transduction is obtained around the injection points. When Ad vectors carry a transgene that exerts cytotoxic effects on transduced cells, Ad vector-mediated hepatic transduction leads to severe hepatotoxicity.[5–7]

In contrast, human species B Ad serotype 35 (Ad35) vectors, which our group and several others have developed,[8–11] possess attractive properties that can overcome the drawbacks of conventional Ad5 vectors. First, Ad35 vector-mediated transduction is not hampered by anti-Ad5 antibodies, because Ad35 belongs to a different species (species B) than Ad5 (species C). Second, Ad35 vectors bind to human CD46 as a receptor.

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