質問 2 細胞基材に対するマイコプラズマ否定試験におけるPCRの位置づけについて，ご意見を伺いたいと思います．現行のものでは，培養法とDNA染色法とPCR法が3法あり，PCR法は培養法が陰性でDNA染色法が陽性になったときに，その確認のために使えるといった位置づけになっています．この試験法が導入されたころのPCRは確か非常に曖昧なところがあったと思いますが，現在PCRは，感度，精度も相当よくなりました．今後再生医療などのように非常にスピーディに結果を出さなければならないときはPCRに頼ってしまうと思います．そういう意味でPCRをあまり継子扱いせず，第1法でどちらでも選択できるような位置づけにした方が，ユーザーとしてはありがたいと思います．

PCRのプライマーを日局の参考情報としてよいと思いますが，EPのようにあまり指定せずに，このようなマイコプラズマを検出できて，しかもこのくらいの数まで検出できるという記載の方がよいと考えます．

回答 PCRを日局あるいは政府のガイドライン的なものに収載したのは，日本が最初ですので継子扱いにすることはありません．ただし，新規収載当時から様々な議論があり，長所，短所のある検出方法ですので，日局の改正には，慎重にその限界を前提とした判断を下さなければいけないこととなります．ルーチンとして再生医療など様々なケースでPCRでマイコプラズマ否定試験を実施することは運用上構わないと思います．また日本薬局方では，規定された方法より良い方法と検証されていれば使用できることになっているので，そのようなプライマーや方法であることが示されれば，もちろんそれが使えます．設定した当初は，FDAなどはけんもほろろで，PCRなんて信用できないと言っていました．培養法やDNA染色法は昔から行われている方法ですし，特に我が国の衛研のセルバンクなどはDNA染色法がお勧めの方法との意見でした．

このような状況で現在に至っていますが，薬局方の規定の中でどのようにPCRを位置づけるか，より合理的に活用できるかなどについて，使い方，プライマー，あるいは試験条件など様々な要素を勘案しつつ，検討して行きたいと思っています．

文　献

1) 厚生労働省：日本薬局方の一部を改正する件，厚生労働省告示第316号，平成19年9月28日．
2) 厚生労働省：日本薬局方の一部を改正する件，厚生労働省告示第425号，平成21年9月30日．

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SHORT COMMUNICATION

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

F Sakurai[1], S-i Nakamura[2,3,7], K Akitomo[1], H Shibata[2], K Terao[2], K Kawabata[1], T Hayakawa[4,5] and H Mizuguchi[1,6]

[1]*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki City, Osaka, Japan;* [2]*Tsukuba Primates Research Center, National Institute of Biomedical Innovation, Tsukuba City, Ibaraki, Japan;* [3]*The Corporation for Production and Research of Laboratory Primates, Tsukuba City, Ibaraki, Japan;* [4]*Pharmaceuticals and Medical Devices Agency, Chiyoda-Ku, Tokyo, Japan;* [5]*Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan and* [6]*Graduate School of Pharmaceutical Sciences, Osaka University, Suita City, Osaka, Japan*

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.

Correspondence: Dr H Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan.
E-mail: mizuguch@nibio.go.jp
[7]Current address: Research Center of Animal Life Science, Shiga University of Medical Science, Otsu City, Shiga, Japan.
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Human CD46 is expressed on almost all human cells, leading to broad tropism of Ad35 vectors in human cells, including coxsackievirus-adenovirus receptor-negative cells.[8,12] However, intravenous administration of Ad35 vectors resulted in inefficient transduction in the organs of human CD46-transgenic (CD46TG) mice and cynomolgus monkeys, which express CD46 in a pattern similar to that of humans.[13–15] These results indicate that CD46 does not successfully serve as a receptor for intravascularly injected Ad35 vectors and that Ad35 vectors are unsuitable for intravascular transduction. However, this property of Ad35 vectors would suggest a potential advantage, in that unwanted transduction would not occur in organs other than the organs targeted following direct injection of Ad35 vectors when draining from injected sites into the bloodstream. These properties suggest that Ad35 vectors would be suitable for gene transfer by local administration into the organs. In this study, we examined the transduction properties of Ad35 vectors following intraorgan administration in nonhuman primates, that is, cynomologus monkeys.

A previously constructed Ad35 vector expressing β-galactosidase (Ad35LacZ)[15] was locally administered at a dose of 1.5×10^{11} vector particles (VP) per point (high dose) or 3×10^{10} VP per point (low dose) in the following eight organs of two cynomolgus monkeys (designated no. 8 and no. 9; no. 8 received the high dose of Ad35LacZ and no. 9 received the low dose): liver, cerebrum, eyeball (vitreous body), quadriceps femoris muscle, pancreas, kidney, spleen and nasal cavity. Four days after administration, the tissues around the injection sites (approximately $40 \times 40 \times 10$ mm^3 with a central focus at the injection point) were collected and subjected to an analysis of β-galactosidase expression and histological pathology. The health condition of the monkeys was also monitored until necropsy.

Overall, both monkeys did well during the experiment. There were no apparent abnormalities in body temperature or heart rate, although no. 8, the high-dose monkey, exhibited slight reductions in blood pressure and body weight. Both monkeys apparently exhibited increased serum levels of aspartate aminotransferase and creatine phosphokinase on days 0–2 after injection. Mild decreases in hemoglobin levels and increases in levels of lactate dehydrogenase and C-reactive protein were also found in both animals. However, these changes were probably due to the operation. The levels of alanine aminotransferase, alkaline phosphatase, albumin, glucose, calcium, chloride and sodium in the serum were mostly within the normal ranges.

After the direct injection of the Ad35 vectors, the transduction profiles were assessed by immunostaining of β-galactosidase in the tissue sections; Table 1 summarizes the results. A detailed transduction profile in each organ is described below.

Liver

Direct injection of Ad35LacZ to the liver caused tissue damage around the injection site (Figures 1a and b). Infiltration of inflammatory cells, necrotic focus and regenerated bile duct epithelial cells were observed. Immunostaining of the liver sections revealed that hepatocytes were mainly transduced with Ad35LacZ in both no. 8 and no. 9 monkeys (Figures 2a and b). A higher level of β-galactosidase was expressed in the liver of no. 8 than in that of no. 9. The transduced cells were predominantly distributed around the injection point (approximately 1×1 mm^2) and were not found outside the periphery of the injection site. β-galactosidase was not expressed in the liver lobes, which were not injected with Ad35LacZ. β-galactosidase-expressing cells were mainly found on the border region between the normal and damaged areas. Direct injection of naked plasmid DNA or Ad5 vectors into mouse liver also resulted in the localized distribution of transgene-expressing cells around the injection points.[16,17] The liver would not allow dispersion of locally injected Ad vectors in the tissue.

Table 1 β-galactosidase expression in the organs following direct injection of Ad35LacZ into organs

	No. 8 (high dose)	*No. 9 (low dose)*
Liver	+++	+
Cerebrum	+++	+
Eyeball	+	−
Femoral muscle	+	+
Pancreas	−	++
Kidney	−	++
Spleen	−	−
Nasal cavity	−	−

+++, strong positive; ++, moderate positive; +, weakly positive; −, negative.

Cerebrum

Ad35LacZ was stereotaxically injected into the left frontal lobe of the cerebrum. After infusion of the high dose of Ad35LacZ, softening of the tissue, which appeared necrotic, was widely observed in the left basal ganglia (Figure 1c). Neutrophils were infiltrated into the necrotic area. In contrast, injection of a low dose of Ad35LacZ resulted in no apparent toxicity, although slight bleeding was found around the artery (Figure 1d). Transduced cells, which appeared to be microglia, were found around the softening regions of both no. 8 and no. 9 animals, although the latter had fewer transduced microglia (Figures 2c and d). There were no β-galactosidase-expressing cells in the right hemisphere of the brain, which was infused with phosphate-buffered saline buffer (data not shown).

Eye

Ad35LacZ was infused into the vitreous body for inoculation into the eyeball. The high dose induced invasion by inflammatory cells, including macrophages and neutrophils, into the ciliary body, iris and retina (Figure 1e). Necrotic changes were also found in all layers of the retina. The low dose caused similar damage to the eyeball. The high dose mediated transduction in the conjunctival epithelial cells (Figure 2e). β-galactosidase expression was not observed in other areas. After injection into the vitreous body, Ad35LacZ might be drained from it and transduce the conjunctival epithelial cells. Bora *et al*.[18] demonstrated that human CD46 was hardly expressed in eye tissues, suggesting that these tissues are refractory to Ad35 vectors. We did not find β-galactosidase expression in the eye of no. 9 animal. Phosphate-buffered saline injection did not result in

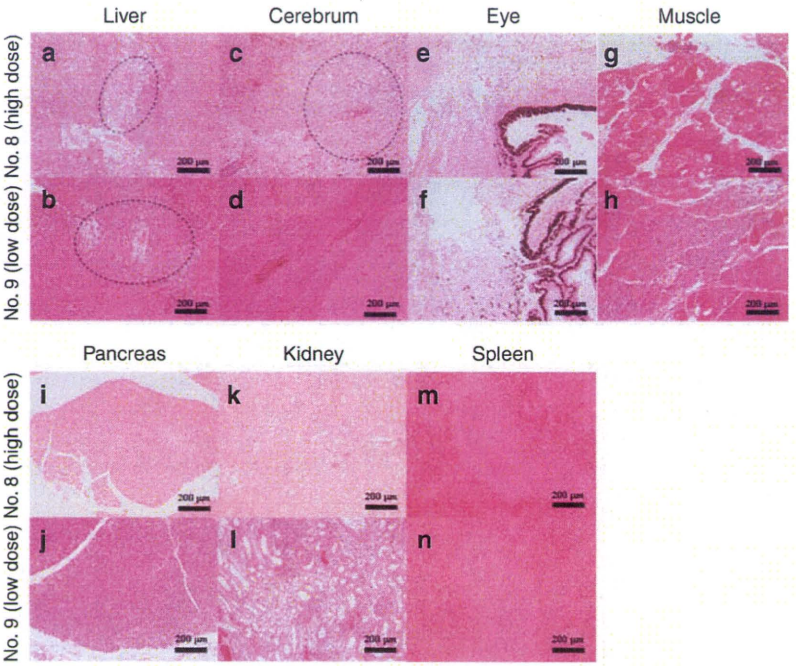

Figure 1 Tissue histology in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and j) pancreas, (**k** and l) kidney and (**m** and **n**) spleen. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and with the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (approximately 3 years of age, 1.9 and 2.2 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan). The liver, cerebrum, eyeball, nasal cavity, pancreas, kidney, skeletal muscle and spleen of cynomolgus monkeys were each injected with Ad35LacZ suspended in 200 μl (100 μl for eyeball) of phosphate-buffered saline at a dose of 1.5×10^{11} vector particles (VP) per point (monkey no. 8) or 3×10^{10} VP per point (monkey no. 9). Four days after injection, tissue sections were hematoxylin–eosin stained by a routine method. Dotted-line circles in (**b**) and (**c**) indicate the necrotic area in the liver and the softening area in the cerebrum, respectively.

transgene expression or apparent abnormality in the eyeball (data not shown).

Femoral muscle

Severe inflammation did not occur after intramuscular injection of the high dose, although we found slight damage to the muscle fibers (Figure 1g). In contrast, the low dose induced more severe inflammation (Figure 1h). Infiltration of neutrophils and macrophages was seen in the muscle of no. 9. It is currently unclear why the low dose induced higher levels of damage. A slight difference in the injection point might affect Ad35 vector-induced inflammatory responses in the muscle. β-galactosidase expression was found only in the cells that appeared to be macrophages and/or fibroblasts located among the muscle fibers in both monkeys (Figures 2g and h). No muscle fibers expressed β-galactosidase in either monkey. It remains to be elucidated why intramuscular injection of Ad35 vectors mediated poor transduction in muscle fibers of cynomolgus monkeys. Ad35 vectors transduced the muscle following intramuscular injection in wild-type mice and in CD46TG mice.[12,14] The transduction mechanism and efficiencies of Ad35 vectors in muscle fibers might differ among species, and the muscle of nonhuman primates might be more refractory to transduction than that of rodents. Thirion *et al.*[19] demonstrated that Ad vectors would transduce human, rat and mouse primary muscle cells through different pathways. Danko *et al.*[20] reported that transgene expression levels by intramuscular injection of naked DNA were lower in dogs and nonhuman primates than in rodents. On the other hand, several studies demonstrated the utility of Ad35 vectors as vaccine vectors that express antigen by intramuscular administration in mice and nonhuman primates.[3,4] Macrophages and/or dendritic cells transduced with Ad35 vectors might play important roles in transgene-specific immune responses by intramuscular injection of Ad35 vectors.

Pancreas

Injection into the pancreas caused no severe damage to that organ in either monkey (Figures 1i and j). We did not find transduced cells in the pancreas of no. 8; in contrast, β-galactosidase was apparently expressed in exocrine acinar cells of no. 9 in the pancreatic lobules (Figures 2i and j). Chemiluminescence assay of β-galactosidase also revealed significant levels of β-galactosidase expression in the pancreas of no. 9 but not in that of no. 8 (data not shown). Wang *et al.*[21] also demonstrated that direct injection of conventional Ad vectors and adenoassociated virus vectors into murine pancreas achieved efficient transduction in acinar cells. Pancreatic acinar cells would be susceptible to Ad vectors.

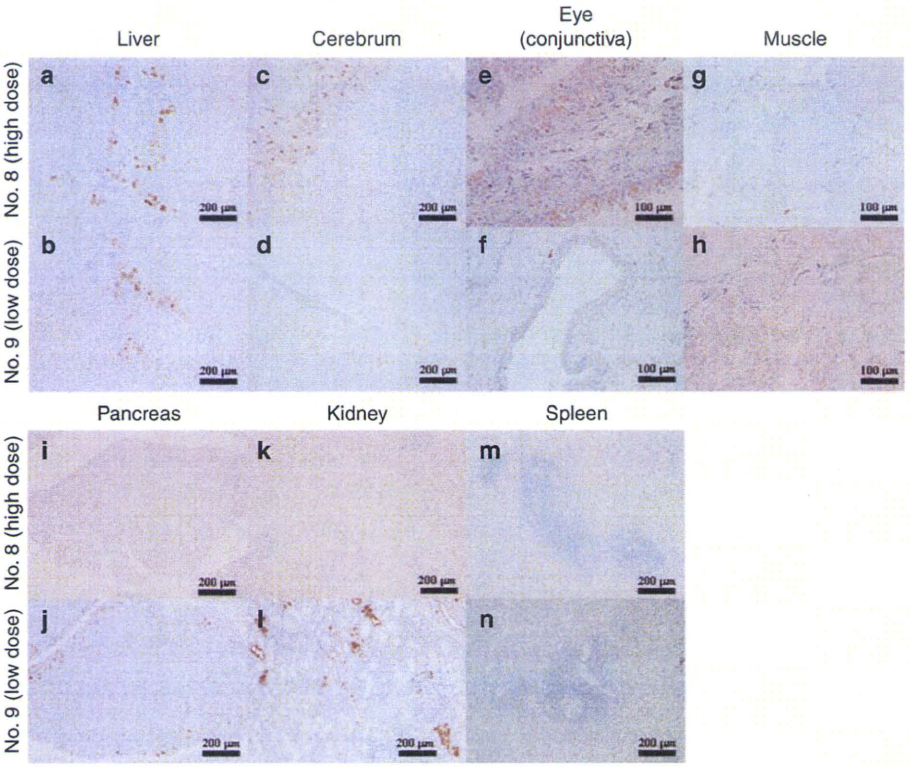

Figure 2 β-galactosidase expression in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point) or high dose (1.5×10^{11} VP per points) as described in Figure 1. Four days after injection, the tissues were collected for analysis of β-galactosidase expression and histological pathology. Immunostaining of β-galactosidase was performed using anti-β-galactosidase antibody (Abcam, Cambridge, UK).

Kidney

Ad35LacZ injection to the left kidney induced infiltration by inflammatory cells, including lymphocytes, into the interstitial tissue of the kidney (Figures 1k and l). The right kidney, which was injected with phosphate-buffered saline, did not exhibit β-galactosidase expression or inflammatory responses (data not shown). The high dose did not mediate β-galactosidase expression, but the low dose led to apparent transduction (Figures 2k and l). The renal tubular epithelial cells were mainly transduced with Ad35LacZ. In the kidney, compared with the other organs, transduced cells were more widely spread around the injection points. Refractoriness to the high dose and massive β-galactosidase expression by the low dose in the pancreas and kidney together form a major conundrum in this study. The differences in transduction efficiencies might be due to the slight differences in injection sites. Especially, Ad35LacZ may have been drained into the renal tubule of no. 9 following injection into the kidney, leading to efficient transduction in the renal tubule epithelial cells. Ad35 was originally identified in the kidney and causes cystitis,[22] indicating the tropism of Ad35 for renal epithelial cells.

Spleen and nasal cavity

Unexpectedly, direct injection of Ad35LacZ to the spleen did not induce inflammatory responses such as hyperplasia (Figures 1m and n). There was no β-galactosidase expression in the spleen of either monkey (Figures 2m and n). For transduction in the mucosal membrane of the nasal cavity, Ad35 vector suspensions were instilled into the nasal cavity of each monkey, but neither one showed β-galactosidase expression or cellular damage in the mucosal membrane of the nasal cavity (data not shown).

Other organs

β-galactosidase production in the lung, heart, thymus, bone marrow, lymph node, bladder and testis, which were not injected with Ad35LacZ, were examined by chemiluminescence assay. None of these organs showed detectable β-galactosidase expression (data not shown).

Next, we determined the blood concentrations of Ad35LacZ genome DNA in the blood using quantitative real-time PCR to examine whether or not Ad35LacZ locally injected to the organs was drained from the injection site into the bloodstream. The Ad35 vector DNA was detected in the blood as soon as 6 h post-injection, then gradually decreased (Figure 3). However, the blood-clearance kinetics of Ad35LacZ following intraorgan injection were slower than those following intravenous administration, which were previously reported,[23] although the total amounts of Ad35 vector doses in this study (no. 8: 1.5×10^{11} VP × 8 points; no. 9: 3×10^{10} VP × 8 points) were comparable to or lower than those in the previous study in which Ad35LacZ was intravenously infused in cynomolgus monkeys ($0.4–2 \times 10^{12}$ VP per kg, 1.88–2.96 kg).[23] Ad35 vector

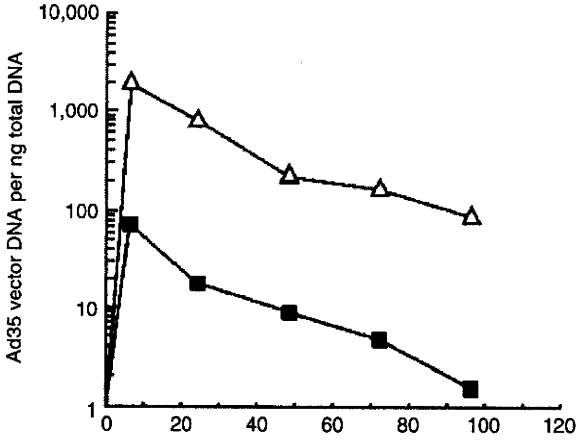

Figure 3 Blood concentrations of Ad35 vectors in cynomolgus monkeys following intraorgan administration. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point, closed square) or high dose (1.5×10^{11} VP per points, open triangle) as described in Figure 1. Blood was collected at the indicated post-injection time points (6, 24, 48, 72 and 96 h post-injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured by quantitative TaqMan PCR assay, as previously described.[23]

DNA was still detectable 4 days after injection. These results suggest that Ad35 vectors or Ad35 vector DNA remaining in the injection sites might be gradually released from the injection sites and drained into the bloodstream.

Furthermore, to examine whether or not Ad35LacZ draining into the bloodstream was accumulated in the organs, we determined the Ad35 DNA contents in the portions of the liver and spleen that were away from the respective injection sites. The liver and spleen play crucial roles in the clearance of systemically injected Ad vectors. The Ad35 vector DNA was not detected in those portions of the liver in no. 9, but was detected in the portions of the liver in no. 8 and in those of the spleen in both monkeys (data not shown). These results suggest that Ad35LacZ or the Ad35 vector DNA draining into the systemic circulation would be taken up by the liver and spleen. We further assessed the Ad35 DNA contents in the lungs, heart, thymus and bone marrow, in which Ad35 vectors were not directly infused. Ad35 vector DNA was detected in the lungs and heart of no. 8 but not in those of no. 9 (data not shown). We did not detect Ad35 vector DNA in the thymus or bone marrow of either monkey. Considering that intravascularly injected Ad35 vectors did not efficiently transduce organs,[15] organs must not be transduced with Ad35LacZ, which is drained into the bloodstream and taken up by the organs.

In most cases of cancer gene therapy using Ad vectors, the vectors are administered directly to the tumor regions.[24–26] When used as vaccine vectors, on the other hand, Ad vectors are intramuscularly injected.[27,28] In addition, Ad vectors are intramyocardially injected in angiogenic gene therapy.[29,30] Thus, direct infusion of Ad vectors to organs is one of the most frequent application methods in clinical settings. However, there has been little information about the transduction properties of these vectors following direct injection into organs. This study demonstrated that different types of cells were transduced with Ad35 vectors depending on the organ after direct infusion into the organ. The differences in the histological structures and cell types comprising the organs would explain the differences in transduction properties of locally injected Ad35 vectors. This study provides important information for clinical study by intraorgan injection of Ad35 vectors, although the characteristics of the organs (structure, cell types and so on) differ different between normal tissue and diseased areas.

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Efficient osteoblast differentiation from mouse bone marrow stromal cells with polylysin-modified adenovirus vectors

Katsuhisa Tashiro [a,b], Asami Kondo [a], Kenji Kawabata [a], Haruna Sakurai [a,b], Fuminori Sakurai [a], Koichi Yamanishi [b,c], Takao Hayakawa [d,e], Hiroyuki Mizuguchi [a,b,*]

[a] Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[b] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[d] Pharmaceutics and Medical Devices Agency, 3-3-2, Kasumigaseki, Chiyoda-Ku, Tokyo 100-0013, Japan
[e] Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1. Kowakae, Higashi-Osaka, Osaka 577-8502, Japan

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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

* Corresponding author. Address: Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Fax: +81 72 641 9816.
E-mail address: mizuguch@nibio.go.jp (H. Mizuguchi).

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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

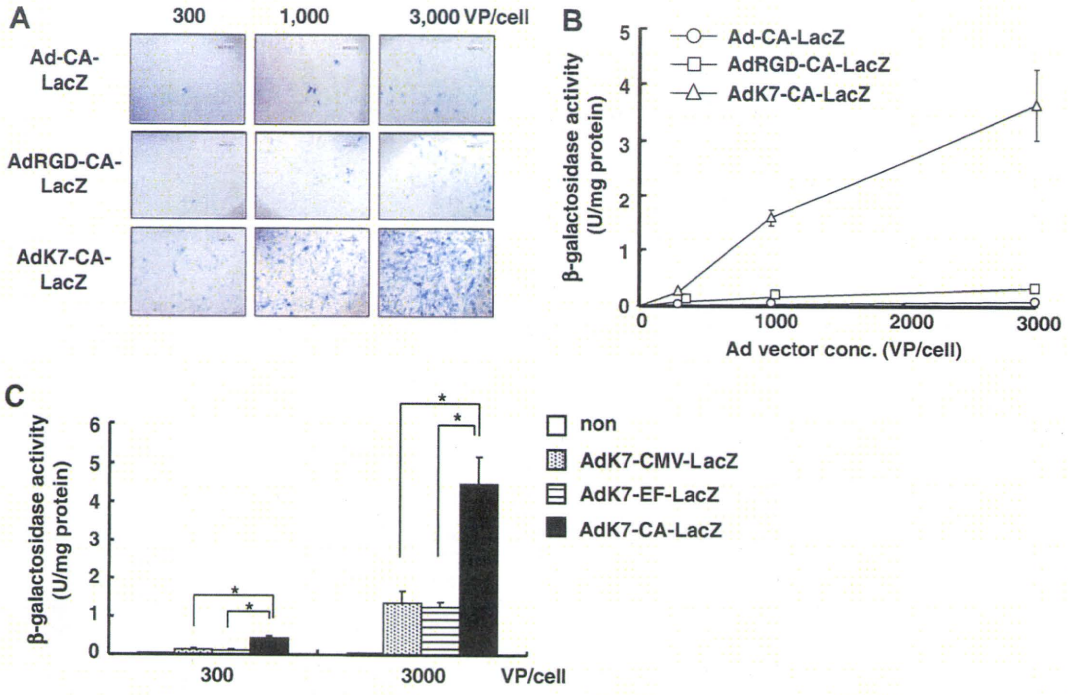

Fig. 1. Gene transduction efficiency in mouse primary BMSCs by various types of Ad vectors. Mouse BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors. Two days later, (A) X-gal staining and (B) luminescence assay were performed. Similar results of X-gal staining were obtained 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).

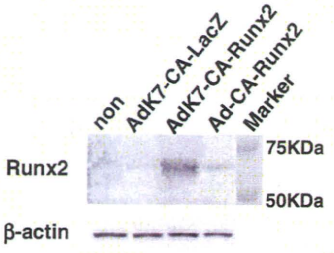

Fig. 2. Runx2 expression in Ad vector-transduced BMSCs. Cell lysates were isolated from BMSCs 2 days after the transduction, and Western blotting was performed.

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

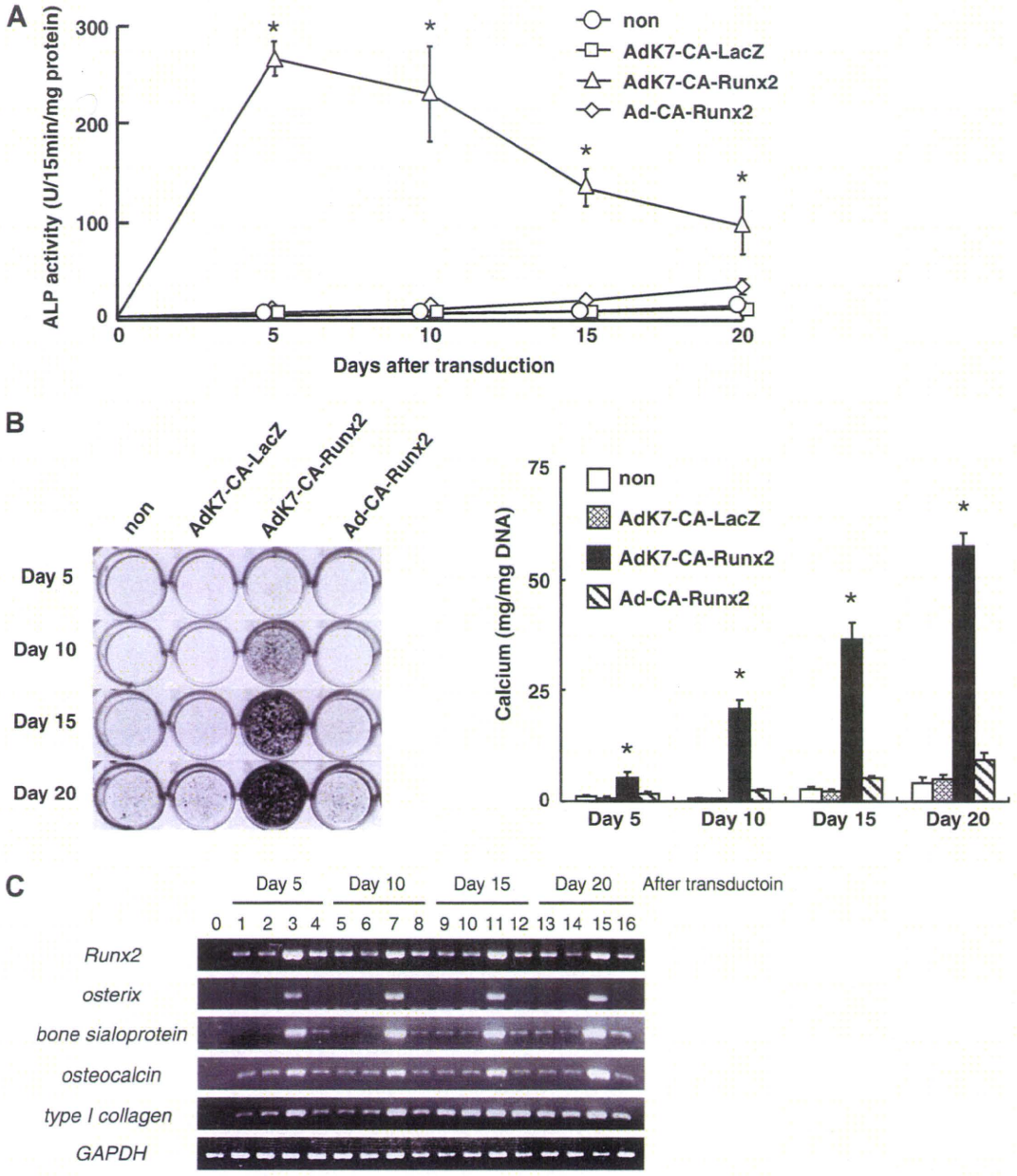

Fig. 3. Promotion of *in vitro* osteoblastic differentiation in AdK7-CA-Runx2-transduced BMSC. After transduction with each Ad vector at 3000 VP/cell for 1.5 hr, BMSCs were cultured for the indicated number of days. (A) ALP activity, (B, left) matrix mineralization, and (B, right) calcium deposition 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

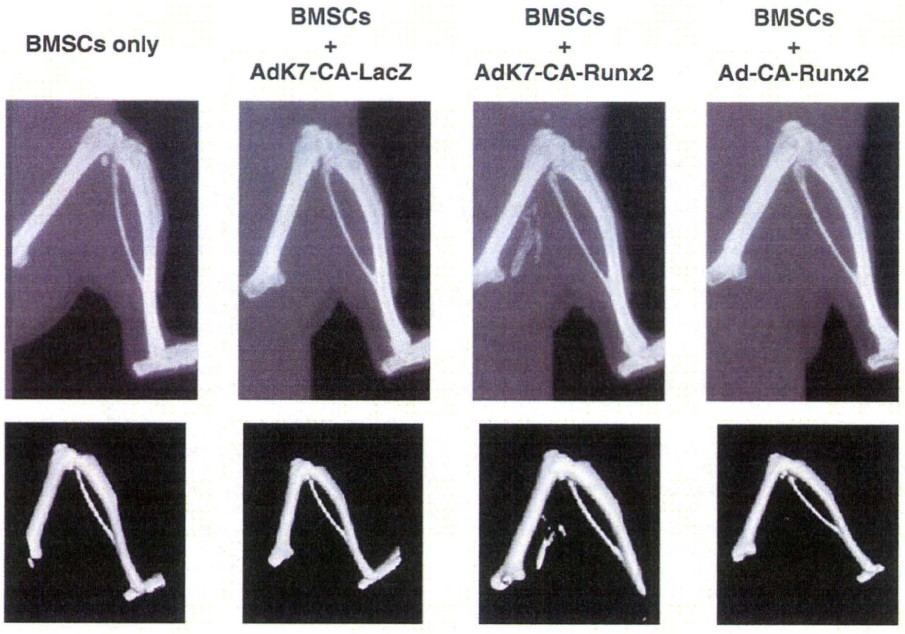

Fig. 4. *In vivo* ectopic bone formation of mouse BMSCs by AdK7-mediated Runx2 gene transduction. BMSCs were transduced with indicated Ad vectors at 3000 VP/cell. On the following day, cells were injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by 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.

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STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Efficient Adipocyte and Osteoblast Differentiation from Mouse Induced Pluripotent Stem Cells by Adenoviral Transduction

KATSUHISA TASHIRO,[a,b] MITSURU INAMURA,[a,b] KENJI KAWABATA,[b] FUMINORI SAKURAI,[b] KOICHI YAMANISHI,[a,c] TAKAO HAYAKAWA,[d,e] HIROYUKI MIZUGUCHI[a,b]

[a]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [b]Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan; [c]National Institute of Biomedical Innovation, Osaka, Japan; [d]Pharmaceutics and Medical Devices Agency, Tokyo, Japan; [e]Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan

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ABSTRACT

Induced pluripotent stem (iPS) cells, which are generated from somatic cells by transducing four genes, are expected to have broad application to regenerative medicine. Although establishment of an efficient gene transfer system for iPS cells is considered to be essential for differentiating them into functional cells, the detailed transduction characteristics of iPS cells have not been examined. Previously, by using an adenovirus (Ad) vector containing the elongation factor-1α (EF-1α) and the cytomegalovirus enhancer/β-actin (CA) promoters, we developed an efficient transduction system for mouse embryonic stem (ES) cells and their aggregate form, embryoid bodies (EBs). In this study, we applied our transduction system to mouse iPS cells and investigated whether efficient differentiation could be achieved by Ad vector-mediated transduction of a functional gene. As in the case of ES cells, the Ad vector containing EF-1α and the CA promoter could efficiently transduce transgenes into mouse iPS cells. At 3,000 vector particles/cell, 80%–90% of iPS cells expressed transgenes by treatment with an Ad vector containing the CA promoter, without a decrease in pluripotency or viability. We also found that the CA promoter had potent transduction ability in iPS cell-derived EBs. Moreover, exogenous expression of a *PPARγ* gene or a *Runx2* gene into mouse iPS cells by an optimized Ad vector enhanced adipocyte or osteoblast differentiation, respectively. These results suggest that Ad vector-mediated transient transduction is sufficient to increase cellular differentiation and that our transduction methods would be useful for therapeutic applications based on iPS cells. STEM CELLS *2009;27:1802–1811*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Because embryonic stem (ES) cells, derived from the inner cell mass of mammalian blastocysts, can be cultured indefinitely in an undifferentiated state and differentiate into various cell types [1, 2], ES cells have been regarded as a potential source of specific cell populations for cell replacement therapy. However, there are two important issues that must be addressed before ES cells can be applied for regenerative medicine: one is the ethical issue about the use of embryos, and the other is the risk of immune rejection after transplantation. In 2006, Takahashi and Yamanaka [3] reported that ES cell-like pluripotent cells, designated as induced pluripotent stem (iPS) cells, could be generated from mouse skin fibroblasts by retroviral transduction of four genes (POU domain class 5 transcription factor 1 [*Oct-3/4*], SRY-box containing box 2 [*Sox2*], cellular myelocytomatosis oncogene [c-*Myc*], and Kruppel-like factor 4 [*Klf4*]). A recent study demonstrated that iPS cells possessed mostly the same characteristics as ES cells, such as global gene expression [4], DNA methylation [5], and histone modification [6]. Furthermore, iPS cells give rise to adult chimeric offspring and show competence for germline transmission [4–6]. Because iPS cells not only have the properties as described above but also can overcome the ethical concerns and problems with immune rejection and because human iPS cells can also be generated from somatic cells [7–10], they are expected to be applicable to regenerative medicine in place of ES cells.

To apply iPS cells to regenerative medicine, establishing methods for the differentiation of iPS cells into pure functional cells is indispensable. Among the many methods for promoting cellular differentiation, genetic manipulation is one of the most powerful techniques, because overexpression of a

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Correspondence: Hiroyuki Mizuguchi, Ph.D., Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Telephone: +81-6-6879-8185; Fax: +81-6-6879-8185; e-mail: mizuguch@phs.osaka-u.ac.jp Received November 25, 2008; accepted for publication April 23, 2009; first published online in STEM CELLS *EXPRESS* April 30, 2009. 1066-5099/2009/$30.00/0 doi: 10.1002/stem.108

differentiation-associated gene in the cells is considered to direct the cell fate from stem cells into functional cells. Many studies have reported that gene transfer into stem cells promoted their differentiation into functional differentiated cells, including hematopoietic cells [11], pancreatic cells [12], and neurons [13].

Adenovirus (Ad) vectors are some of the most efficient gene delivery vehicles and have been widely used in both experimental studies and clinical trials [14, 15]. Ad vectors are an attractive vehicle for gene transfer because they are easily constructed, can be prepared in high titers, and provide efficient transduction in both dividing and nondividing cells. We have developed efficient methods for Ad vector-mediated transduction into mouse ES cells and their aggregate form, embryoid bodies (EBs) [16, 17]. We also showed that adipocyte differentiation from mouse ES cells was markedly promoted by use of the Ad vector for transient transduction of the peroxisome proliferator-activated receptor γ (*PPAR*γ) gene [17], which is known to play essential roles in adipogenesis [18, 19].

Because our transduction method using an optimized Ad vector was effective for enhancing the differentiation of mouse ES cells into target cells, we attempted to apply this system to mouse iPS cells and examined whether the adipocyte and osteoblast differentiation potential of mouse iPS cells could be increased by using Ad vector. In all studies, mouse ES cells were used as a control for comparison with mouse iPS cells. By comparing the promoter activity in mouse iPS cells, we successfully developed a suitable Ad vector for gene transfer into mouse iPS cells. We also found that adipocyte and osteoblast differentiation from mouse iPS cells could be facilitated by Ad vector-mediated transient transduction of a *PPAR*γ gene and a runt-related transcription factor 2 (*Runx2*) gene, respectively.

Materials and Methods

Adenovirus Vectors

Ad vectors were constructed by an improved in vitro ligation method [20, 21]. The shuttle plasmids pHMCMV5, pHMCA5, and pHMEF5, which contain the cytomegalovirus (CMV) promoter, the CMV enhancer/β-actin promoter with β-actin intron (CA) promoter (a kind gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan) [22], and the human elongation factor-1α (EF-1α) promoter, respectively, were constructed previously [16, 21]. The *mCherry* gene, which is derived from pmCherry (Clontech, Mountain View, CA, http://www.clontech.com), was inserted into pHMCMV5, pHMCA5, and pHMEF5, resulting in pHMCMV-mCherry, pHMCA-mCherry, and pHMEF-mCherry, respectively. pHMCMV-mCherry, pHMCA-mCherry, or pHMEF-mCherry was digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested pAdHM4 [20], resulting in pAd-CMV-mCherry, pAd-CA-mCherry, or pAd-EF-mCherry, respectively. Ad-CMV-mCherry, Ad-CA-mCherry, and Ad-EF-mCherry were generated and purified as described previously [17]. The Rous sarcoma virus (RSV) promoter-, the CMV promoter-, the CA promoter-, or the EF-1α promoter-driven β-galactosidase (LacZ)-expressing Ad vector (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ, respectively), the CA promoter-driven mouse PPARγ2-expressing Ad vector (Ad-CA-PPARγ2), the CA promoter-driven mouse Runx2-expressing Ad vector (Ad-CA-Runx2), and a transgene-deficient Ad vector (Ad-null), were generated previously [16, 17, 23, 24]. The vector particle (VP) titer and biological titer were determined by using a spectrophotometric method [25] and by means of an Adeno-X Rapid Titer Kit (Clontech), respectively. The ratios of the biological-to-particle titer were 1:31 for Ad-CMV-mCherry, 1:20 for Ad-CA-mCherry, 1:28 for Ad-EF-mCherry, 1:14 for Ad-CA-LacZ, 1:22 for Ad-EF-LacZ, 1:41 for Ad-RSV-LacZ, 1:21 for Ad-CMV-LacZ, 1:8 for Ad-CA-PPARγ2, 1:17 for Ad-CA-Runx2, and 1:11 for Ad-null.

Mouse ES and iPS Cell Cultures

Three mouse iPS cell clones 20D17, 38C2, and stm99-1 (a kind gift from Dr. S. Yamanaka, Kyoto University, Kyoto, Japan) were used in the present study (20D17 was purchased from Riken BioResource Center, Tsukuba, Japan, http://www.brc.riken.jp) [4, 26]. 20D17 and 38C2, both of which carry Nanog promoter-driven green fluorescent protein (GFP)/internal ribosomal entry site/puromycin-resistant gene, were generated from mouse embryonic fibroblasts (MEFs) [4], and stm99-1, carrying the Fbx15 promoter-driven β-geo cassette (a fusion of the β-galactosidase and neomycin resistance genes), was generated from gastric epithelial cells [26]. These mouse iPS cells and mouse E14 ES cells were routinely cultured in leukemia inhibitory factor-containing ES cell medium (Speciality Media) on mitomycin C-treated MEFs, and iPS cell lines and ES cells were passaged every 2nd day using 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Mouse iPS cells 20D17 and E14 ES cells were also cultured on a gelatin-coated dish. To obtain GFP-expressing undifferentiated cells, iPS cells 20D17 were cultured in ES cell medium containing 1.5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) on a gelatin-coated dish. Mouse iPS cell clone 20D17 was used in this report except where otherwise indicated. EB formation from mouse ES and iPS cells was induced using the hanging drop method as described previously [17].

LacZ Assay

Mouse ES cells or iPS cells (5×10^4 cells) were plated on 24-well plates. On the following day, they were transduced with each Ad vector (Ad-null, Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ) at 3,000 VPs/cell for 1.5 hours. At 24 hours after incubation, X-galactosidase (Gal) staining was performed as described previously [16]. ES cell-derived EBs (ES-EBs) or iPS cell-derived EBs (iPS-EBs) cultured for 5 days (5d-ES-EBs or 5d-iPS-EBs, respectively) were transduced with each Ad vector at 3,000 VPs/cell. Two days later, LacZ expression was measured by X-Gal staining and β-Gal luminescence assays.

mCherry Expression Analysis

Mouse ES cells or iPS cells were plated on gelatin-coated 24-well plates. On the following day, they were transduced with the indicated dose of Ad-CA-mCherry or Ad-EF-mCherry for 1.5 hours. Twenty-four hours later, mCherry expression was analyzed by flow cytometry on an LSR II flow cytometer using FACSDiva software (BD Biosciences, Tokyo, Japan, http://www.bdbiosciences.com). To transduce the EB interior, the ES-EBs or iPS-EBs were transduced with 3,000 VPs/cell of Ad-CMV-mCherry or Ad-CA-mCherry three times on days 0, 2, and 5 (hereinafter referred to as triple transduction) [17]. In brief, 0d-ES-EBs or 0d-iPS-EBs (ES or iPS cell suspension, respectively) were transduced with Ad vector at 3,000 VPs/cell in a hanging drop for 2 days, and 2d-ES-EBs or 2d-iPS-EBs and 5d-ES-EBs or 5d-iPS-EBs were transduced with the same Ad vector at 3,000 VPs/cell for 1.5 hours. On day 7, mCherry expression in the ES-EBs or iPS-EBs was visualized via confocal microscopy (Leica TCS SP2 AOBS; Leica Microsystems, Tokyo, Japan, http://www.leica.com). The ES-EBs or iPS-EBs were then trypsinized and analyzed for mCherry expression by flow cytometry.

Expression of Coxsackievirus and Adenovirus Receptors

For detection of coxsackievirus and adenovirus receptor (CAR) expression, ES and iPS cells, both of which were cultured on gelatin-coated dishes, were harvested by using phosphate-buffered saline (PBS) containing 1 mM EDTA. Cells were then reacted

with rat anti-mouse CAR monoclonal antibody (kindly supplied from Dr. T. Imai, KAN Research Institute, Hyogo, Japan) and stained with phycoerythrin-labeled donkey anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). CAR expression was analyzed by using an LSR II flow cytometer.

In Vitro Differentiation

Two days after culture with a hanging drop, the EBs were transferred into a Petri dish and maintained for 3 days in suspension culture in differentiation medium (Dulbecco's modified Eagle's medium [Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english] supplemented with 15% fetal calf serum [Specialty Media, Inc., Phillipsburg, NJ, http://www.millipore.com], 0.1 mM 2-mercaptoethanol [Nacalai Tesque, Kyoto, Japan, http://www.nacalai.co.jp.en], 1× nonessential amino acid [Specialty Media, Inc.], 1× nucleosides [Specialty Media, Inc.], 2 mM L-glutamine [Invitrogen], and penicillin/streptomycin [Invitrogen]) containing 100 nM all-*trans*-retinoic acid (RA) (Wako Chemical) and then cultured for 2 more days in differentiation medium without RA [27, 28]. The cells were transduced with 3,000 VPs/cell of Ad vector (Ad-CA-LacZ, Ad-CA-PPARγ2, or Ad-CA-Runx2) at days 0, 2, and 5 as described above and plated on a gelatin-coated dish on day 7. For adipogenic or osteoblastic differentiation, cells were cultured in differentiation medium containing adipogenic supplements (0.1 M 3-isobutyl-L-methylxanthine [Sigma-Aldrich], 100 nM insulin [Sigma-Aldrich], 10 nM dexamethasone [Wako Chemical], and 2 nM triiodothyronine [Sigma-Aldrich]) or osteogenic supplements (50 μg/ml ascorbic acid 2-phosphate [Sigma-Aldrich]), 5 mM β-glycerophosphate [Sigma-Aldrich], and 10 nM dexamethasone [Wako Chemical]), respectively.

Biochemical Assays

Cells were cultured with adipogenic or osteogenic supplements for 15 days after plating on gelatin-coated plates. Adipocyte differentiation from mouse ES and iPS cells was evaluated by oil red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity. The oil red O staining and GPDH assay were performed using a Lipid Assay kit and GPDH Assay kit, respectively (Primary Cell Co., Ltd, Hokkaido, Japan, http://www.primarycell.com), according to the manufacturer's instructions. To detect matrix mineralization in the cells, cells were fixed with 4% paraformaldehyde-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. Insoluble material was removed by centrifugation. The supernatants were then assayed for calcium concentration with a calcium C-test kit (Wako Chemical). DNA in pellets was extracted using a DNeasy tissue kit (Qiagen, Valencia, CA, http://www1.qiagen.com), and calcium content was then normalized to cellular DNA. For the measurement of alkaline phosphatase (ALP) activity, 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. ALP activity was measured using the LabAssay ALP kit (Wako Chemical) according to the manufacturer's instructions. The protein concentration of the lysates was determined using a Bio-Rad assay kit (Bio-Rad, Hercules, CA, http://www.bio-rad.com), and ALP activity was then normalized by protein concentration.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from various kinds of cell populations with the use of ISOGENE (Nippon Gene, Tokyo, Japan, http://www.nippongene.com). cDNA was synthesized by using SuperScript II reverse transcriptase (RT) (Invitrogen) and the oligo(dT) primer. Polymerase chain reaction (PCR) was performed with the use of KOD Plus DNA polymerase (Toyobo, Osaka, Japan, http://www.toyobo.co.jp/e). The product was assessed by 2% agarose gel electrophoresis followed by ethidium bromide staining. The sequences of the primers used in this study are listed in supporting information Table S1.

Teratoma Formation and Histological Analysis

Mouse iPS cells were transduced with Ad-CA-mCherry at 10,000 VPs/cell for 1.5 hours. After culture for 3 days, mouse iPS cells were suspended at 1×10^7 cells/ml in PBS. Nude mice (8-10 weeks; Nippon SLC, Shizuoka, Japan, http://www.jslc.co.jp) were anesthetized with diethyl ether, and we injected 100 μl of the cell suspension (1×10^6 cells) subcutaneously into their backs. Five weeks later, tumors were surgically dissected from mice. Samples were washed, fixed in 10% formalin, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with hematoxylin and eosin. This process was commissioned to Applied Medical Research Laboratory (Osaka, Japan).

RESULTS

Mouse iPS Cells Express Coxsackievirus and Adenovirus Receptor

In the present study, we mainly used the mouse iPS cell clone 20D17 [4]. To assess whether iPS cells have properties similar to those of ES cells under the present culture conditions, we initially investigated the expression of cellular marker genes of iPS cells (Fig. 1A). Semiquantitative RT-PCR analysis revealed that Oct-3/4 and Nanog, both of which are undifferentiated markers in ES cells, were strongly expressed in iPS cells. iPS cells also expressed GFP in the undifferentiated state only, because GFP expression was driven by the Nanog promoter [4]. By EB formation, the expression levels of Oct-3/4, Nanog, and GFP in iPS cells were decreased and, in turn, the three germ layer marker genes (ectoderm: nestin and fibroblast growth factor-5; mesoderm: brachyury T and flk-1; and endoderm: GATA-binding protein-6 and α-fetoprotein) were expressed. These results showed that the gene expression patterns of iPS cells were indistinguishable from those of ES cells.

We next examined the expression of CAR, a primary Ad receptor on the cellular surface, in iPS cells, because the expression of CAR is known to be essential for the transduction using the conventional Ad vector [29–31]. We have reported that CAR was highly expressed in mouse ES cells and ES-EBs [16, 17]. RT-PCR and flow cytometric analysis showed that CAR expression was observed in iPS cells and the expression level of CAR in iPS cells and iPS-EBs was equivalent to that in ES cells and ES-EBs, respectively (Fig. 1A, 1B). Notably, the expression of CAR was observed in more than 95% of GFP-expressing undifferentiated iPS cells. These results suggest that iPS cells could be efficiently transduced by using a conventional Ad vector.

Ad Vectors Containing the CA or the EF-1α Promoter Have Potent Transduction Activity in Mouse iPS Cells

To examine the transduction efficiency in iPS cells by using Ad vectors, we 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). We also prepared Ad-null, a transgene-deficient Ad vector, as a control vector. ES and iPS cells were transduced with each Ad vector at 3,000 VPs/cell, and LacZ expression in the cells was measured. X-Gal staining showed that Ad-RSV-LacZ- or Ad-CMV-LacZ-transduced ES cells expressed little LacZ, whereas Ad-CA-LacZ- or Ad-EF-

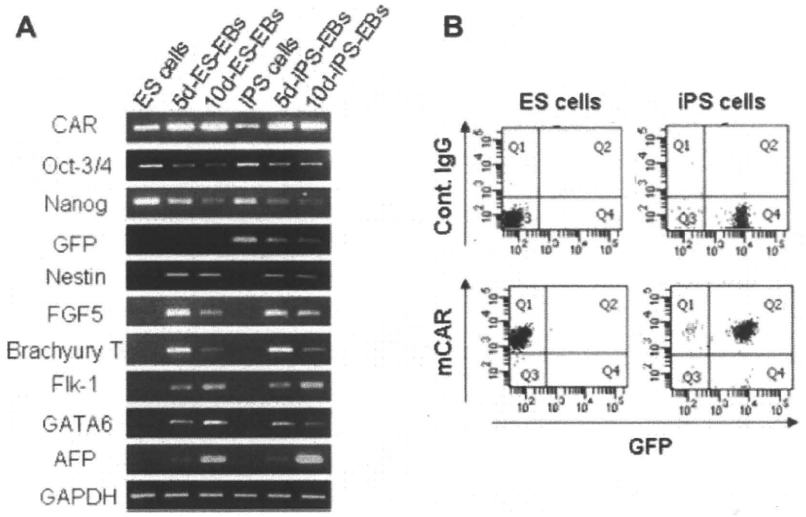

Figure 1. Gene expression patterns of mouse iPS cells were similar to those of mouse ES cells. **(A):** Total RNA was isolated from mouse ES cells (lane 1), 5d-ES-EBs (lane 2), 10d-ES-EBs (lane 3), iPS cells (lane 4), 5d-iPS-EBs (lane 5), or 10d-iPS-EBs (lane 6), and semiquantitative reverse transcriptase-polymerase chain reaction was then performed as described in Materials and Methods. The primers for Oct-3/4 and Nanog amplified both endogenous gene and exogenous factors. **(B):** The expression levels of CAR in mouse ES cells and iPS cells were detected with anti-mouse CAR monoclonal antibody by flow cytometry. As a negative control, the cells were incubated with an irrelevant antibody. Data shown are from one representative experiment of three performed. Abbreviations: AFP, α-fetoprotein; CAR, coxsackievirus and adenovirus receptor; Cont., control; EB, embryoid body; ES, embryonic stem; 5d-ES-EBs, ES cell-derived 5-day-cultured EBs; 10d-ES-EBs, ES cell-derived 10-day-cultured EBs; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA, GATA-binding protein; GFP, green fluorescent protein; iPS, induced pluripotent stem; 5d-iPS-EBs, iPS cell-derived 5-day-cultured EBs; 10d-iPS-EBs, iPS cell-derived 10-day-cultured EBs; mCAR, mouse CAR.

LacZ-transduced ES cells successfully expressed LacZ (Fig. 2A, top) as described previously [16]. Likewise, the CA and the EF-1α promoter but not the RSV or the CMV promoter exhibited potent transduction activity in iPS cells (Fig. 2A, bottom). Besides mouse iPS cell clone 20D17, mouse iPS cell clones 38C2 and stm99-1, which were generated from MEFs [4] and gastric epithelial cells [26], respectively, also efficiently expressed transgenes by an Ad vector containing the CA or EF-1α promoter (supporting information Fig. S1).

To confirm that the transgene was expressed in GFP-expressing undifferentiated iPS cells, we generated Ad-CA-mCherry and Ad-EF-mCherry, both of which express a monomeric DsRed variant, mCherry. Flow cytometric and fluorescent microscopic analysis showed that the mCherry expression was observed in GFP-expressing iPS cells transduced with Ad-CA-mCherry or Ad-EF-mCherry (Fig. 2B, supporting information Fig. S2). Furthermore, the expression of mCherry in iPS cells was dose-dependent, and more than 90% of the cells expressed mCherry after transduction with 10,000 VPs/cell of Ad-CA-mCherry and Ad-EF-mCherry (Fig. 2C and data not shown). Importantly, there was no significant difference in the percentage of GFP-positive cells between nontransduced cells and Ad-CA-mCherry- or Ad-EF-mCherry-transduced cells (Fig. 2D and data not shown). Moreover, neither alkaline phosphatase activity nor Oct-3/4 expression in iPS cells on day 3 after Ad vector-mediated transduction was different from that in nontransduced cells (supporting information Fig. 3). We also examined the pluripotency of Ad vector-transduced iPS cells by teratoma formation. Mouse iPS cells were transduced with Ad vector and were then injected subcutaneously into the backs of nude mice. After subcutaneous transplantation, we obtained teratomas containing epidermis, cartilage, and gut epithelial tissues (Fig. 2E). These observations demonstrated that the undifferentiated state and pluripotency in iPS cells were still maintained even after Ad vector transduction. Furthermore, we counted the number of viable iPS cells at 24, 48, and 72 hours after transduction to investigate the cytotoxicity in iPS cells transduced with Ad-CA-mCherry at 3,000 or 10,000 VPs/cell. The number of viable iPS cells transduced with Ad-CA-mCherry at 3,000 VPs/cell was comparable to the number of viable nontransduced iPS cells, whereas the number of viable iPS cells was slightly (but not significantly) reduced in Ad-CA-mCherry-transduced iPS cells at 10,000 VPs/cell (Fig. 2F). This result was quite similar to that for ES cells (Fig. 2F), and our data suggest that Ad vector transduction has almost no cytotoxicity against either mouse ES cells or mouse iPS cells. These results clearly demonstrated that an Ad vector containing the CA or the EF-1α promoter is an appropriate vector for both ES cells and iPS cells and that iPS cells have the same features as ES cells in terms of Ad vector-mediated transduction.

Ad Vectors Containing the CA Promoter Robustly Drove Transgene Expression in iPS-EBs

We next performed a transduction experiment for ES-EBs and iPS-EBs using a LacZ-expressing Ad vector. Consistent with our previous report [17], the CA promoter showed the highest LacZ expression in ES-EBs. Similarly, the CA promoter showed the highest transduction efficiency in iPS-EBs (Fig. 3A, 3B). Interestingly, the CMV promoter had strong activity in iPS-EBs despite its weak activity in ES cells, ES-EBs, and undifferentiated iPS cells (Figs. 2A, 3A, 3B). These phenomena were also observed by using other iPS cell clone-derived EBs (supporting information Fig. 4).

We next attempted to express the transgene inside the ES-EBs and iPS-EBs, as it is considered to be essential to express the transgene in the EB interior to differentiate ES cells or iPS cells into functional cells. Thus, ES-EBs and iPS-EBs were transduced in triplicate with Ad-CMV-mCherry or Ad-CA-mCherry. This transduction method, namely the triple transduction method, is a gene transfer method that uses an Ad vector to express the transgene in the EB interior (see

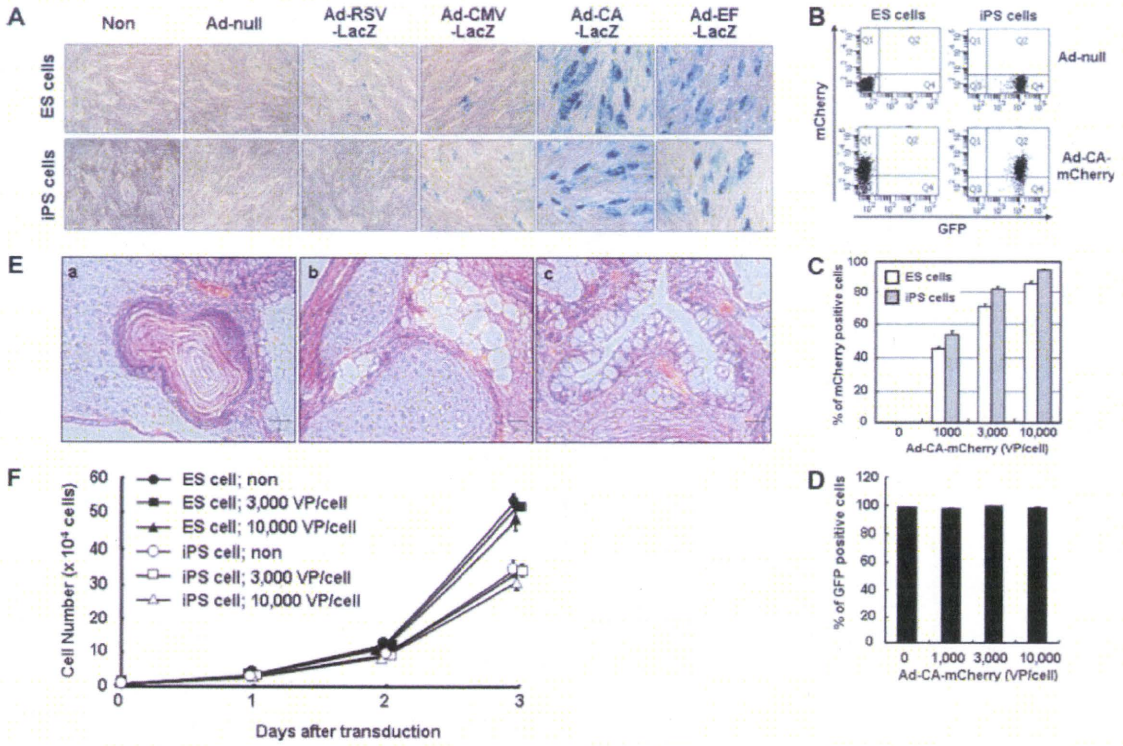

Figure 2. Efficient transgene expression in mouse iPS cells by using an Ad vector containing the CA and the EF-1α promoter. **(A):** Mouse ES cells or iPS cells were transduced with a LacZ-expressing Ad vector at 3,000 VPs/cell. On the following day, X-galactosidase (Gal) staining was carried out. Similar results for X-Gal staining were obtained in three independent experiments. **(B):** Mouse ES cells or iPS cells were transduced with Ad-CA-mCherry at 3,000 VPs/cell, and mCherry-expressing cells were then analyzed by flow cytometry. **(C, D):** Mouse ES cells or iPS cells were transduced with different amounts of Ad-CA-mCherry for 1.5 hours. mCherry expression **(C)** and GFP expression **(D)** were determined by flow cytometry. The data are expressed as the mean ± SD ($n = 3$). **(E):** Paraffin sections of the teratomas derived from Ad-CA-mCherry-transduced iPS cells were prepared, and sections were stained with hematoxylin and eosin: a, ectoderm (epidermis); b, mesoderm (cartilage and adipocyte); c, endoderm (gut epithelium) **(F):** After adenoviral transduction, viable mouse ES cells or iPS cells were counted. Data are expressed as the mean ± SD ($n = 3$). Abbreviations: Ad, adenovirus; CA, cytomegalovirus enhancer/β-actin promoter; CMV, cytomegalovirus; EF, elongation factor-1α; ES, embryonic stem; GFP, green fluorescent protein; IPS, induced pluripotent stem; LacZ, β-galactosidase; RSV, Rous sarcoma virus; VP, vector particle.

Materials and Methods) [17]. Confocal microscopic analysis revealed mCherry expression interior in ES-EBs or iPS-EBs by triple transduction, whereas mCherry expression was observed only in the periphery of the ES-EBs or iPS-EBs by single transduction (Fig. 3C). The percentage of mCherry-positive cells in the ES-EBs or iPS-EBs transduced in triplicate with Ad-CA-mCherry was 43% or 56%, respectively, as determined by flow cytometry (Fig. 3C). In addition, confocal microscopic analysis and flow cytometric analysis showed that Ad-CMV-mCherry-transduced ES-EBs expressed little mCherry even using the triple transduction method, whereas iPS-EBs transduced in triplicate with Ad-CMV-mCherry expressed mCherry only in the periphery of the iPS-EBs. These results are in agreement with LacZ expression in Ad-CMV-LacZ-transduced iPS-EBs as described above. Our data demonstrated that, as in the case of ES cells and ES-EBs, the choice of a suitable promoter was important for efficient transduction in iPS cells and iPS-EBs.

Adipocyte and Osteoblast Differentiation of Mouse iPS Cells Was Facilitated by Ad Vector Transduction

We have shown previously that adipocyte differentiation from mouse ES cells is enhanced by the transduction of the *PPARγ* gene, which is known to be a master regulator gene for adipogenesis [18, 19], into ES cells and ES-EBs using an Ad vector. In this study, to examine whether adipocyte differentiation from iPS cells could also be promoted by Ad vector-mediated transduction and to compare the adipogenic potential between ES cells and iPS cells, both types of cells were differentiated into adipocytes by the transduction of the *PPARγ* gene using the triple transduction method described above. Oil red O staining after culturing for 15 days revealed that lipid droplets were accumulated in both ES cell-derived cells and iPS cell-derived cells by culturing with adipogenic supplements, although the level of lipid accumulation in iPS cell-derived cells was lower than that in ES cells-derived cells (Fig. 4A). In the presence of adipogenic supplements, the percentage of oil red O-positive cells in nontransduced or Ad-CA-LacZ-transduced ES-EBs was approximately 50%, whereas 20%-30% of the nontransduced or Ad-CA-LacZ-transduced iPS-EBs were positive for oil red O. Importantly, adipocyte differentiation in Ad-CA-PPARγ-transduced cells was more efficient than that in nontransduced or Ad-CA-LacZ-transduced cells (Fig. 4A). Oil red O-positive cells in Ad-CA-PPARγ-transduced ES cell- or iPS cell-derived cells were more than 90% or 80% of the total cells, respectively. Furthermore, enhanced adipocyte differentiation from PPARγ-transduced ES and iPS cells was also confirmed by measuring the activity of GPDH and the expression of marker genes

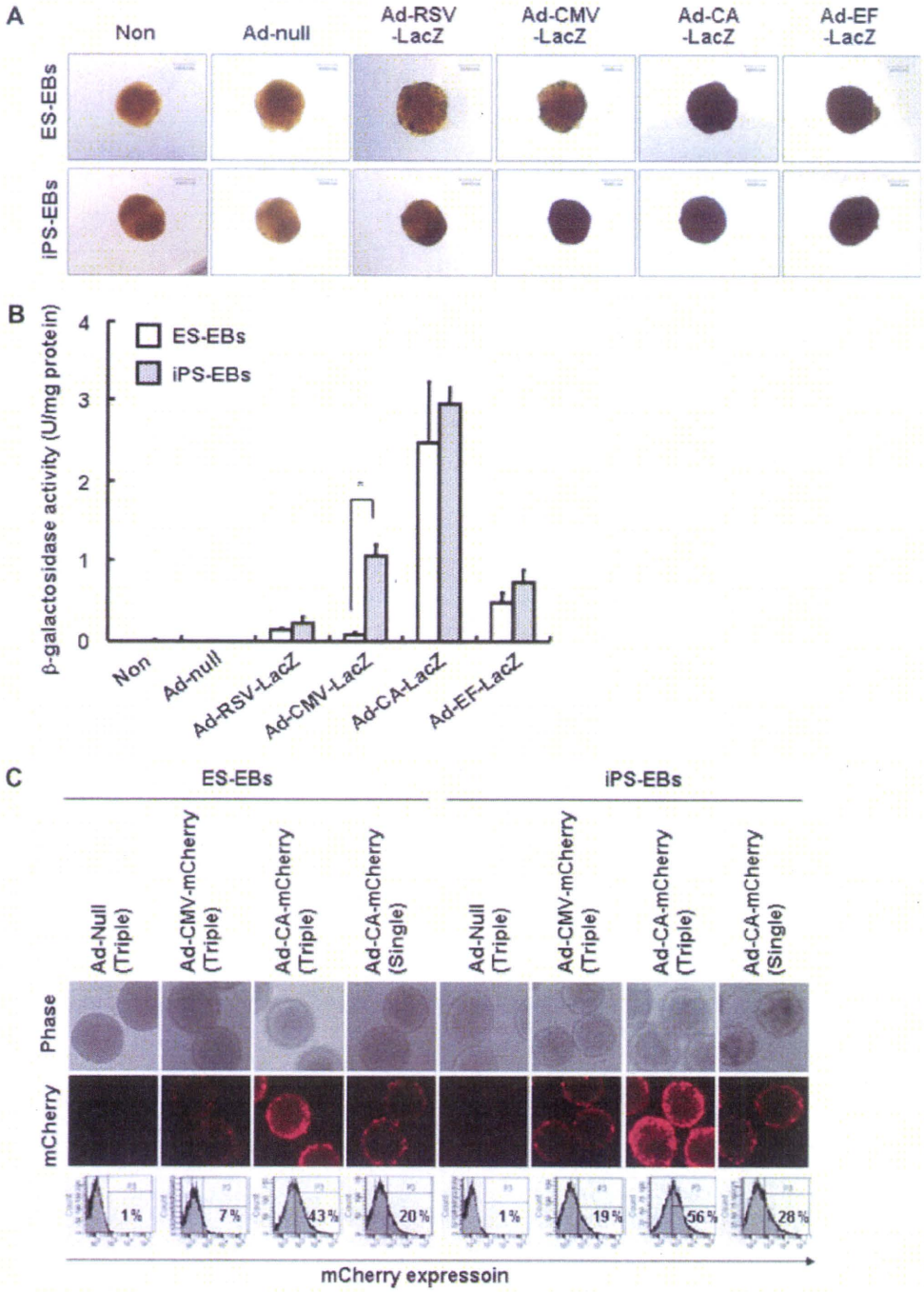

Figure 3. Comparison of promoter activity in iPS-EBs by using Ad vectors. ES cell-derived or IPS cell-derived 5-day-cultured EBs were transduced with each Ad vector at 3,000 vector particles/cell. After 48 hours, X-galactosidase (Gal) staining (**A**) and a β-galactosidase luminescence assay (**B**) were performed as described in Materials and Methods. **(A):** Similar results of X-gal staining were obtained in six independent experiments. **(B):** Data are expressed as the mean ± SD ($n = 3$). *, $p < .01$ **(C):** Either ES-EBs or iPS-EBs was transduced with Ad vectors by triple transduction (Triple) or by single transduction (Single). mCherry expression in ES-EBs or iPS-EBs was detected by confocal microscopy and flow cytometry. As a negative control, both types of EBs were transduced with Ad-null by triple transduction. Abbreviations: Ad, adenovirus; CA, cytomegalovirus enhancer/β-actin promoter; CMV, cytomegalovirus; EB, erythroid body; EF, elongation factor-1α; ES, embryonic stem; IPS, induced pluripotent stem; LacZ, β-galactosidase; RSV, Rous sarcoma virus.

characteristic of adipocyte differentiation (Fig. 4B, 4C). Interestingly, iPS cells were more efficiently differentiated into adipocytes than were ES cells after Ad vector transduction. The GPDH activity in PPARγ-transduced ES cells was twofold higher than that in nontransduced or LacZ-transduced ES cells, whereas PPARγ-transduced iPS cells showed

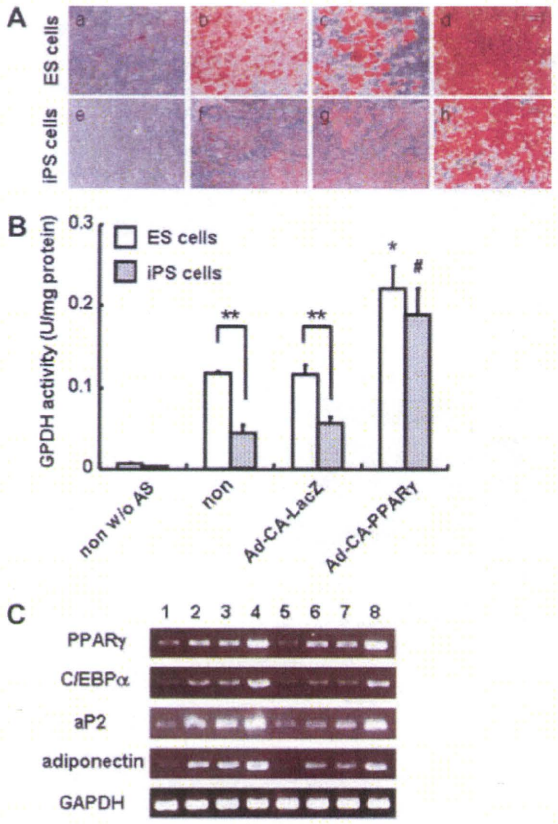

Figure 4. Efficient adipocyte differentiation from mouse ES cells and iPS cells by the transduction of the *PPARγ* gene. ES-EBs or iPS-EBs were transduced in triplicate with 10,000 vector particles/cell of Ad-CA-LacZ or Ad-CA-PPARγ. After plating onto a gelatin-coated dish on day 7, ES-EBs and iPS-EBs were cultured for 15 days in the presence or absence of AS. After cultivation, **(A)** lipid accumulation was detected by oil red O staining, and **(B)** GPDH activity in the cells was measured. **(A):** a, nontreated ES-EBs; b, ES-EBs with AS; c, ES-EBs with AS plus Ad-CA-LacZ; d, ES-EBs with AS plus Ad-CA-PPARγ; e, nontreated iPS-EBs; f, iPS-EBs with AS; g, iPS-EBs with AS plus Ad-CA-LacZ; h, iPS-EBs with AS plus Ad-CA-PPARγ. Scale bar = 60 μm. **(B):** Data are expressed as the mean ± SD (n = 3). *, $p < .01$; **, $p < .05$, compared with nontransduced or Ad-CA-LacZ-transduced ES cells. #, $p < .05$, compared with nontransduced or Ad-CA-LacZ-transduced iPS cells. **(C):** Expression of PPARγ, C/EBPα, aP2, adiponectin, and GAPDH was measured by semiquantitative reverse transcriptase-polymerase chain reaction. Lane 1, nontreated ES-EBs; lane 2, ES-EBs with AS; lane 3, ES-EBs with AS plus Ad-CA-LacZ; lane 4, ES-EBs with AS plus Ad-CA-PPARγ; lane 5, nontreated iPS-EBs; lane 6, iPS-EBs with AS; lane 7, iPS-EBs with AS plus Ad-CA-LacZ; lane 8, iPS-EBs with AS plus Ad-CA-PPARγ. Abbreviations: AD, adenovirus; AS, adipogenic supplements; CA, cytomegalovirus enhancer/β-actin promoter; C/EBPα, CCAAT/enhancer binding protein α; ES, embryonic stem; EB, erythroid body; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; IPS, induced pluripotent stem; LacZ, β-galactosidase; PPARγ, peroxisome proliferator-activated receptor γ; w/o, without.

approximately fourfold higher GPDH activity than nontransduced or LacZ-transduced iPS cells. These results showed that, like ES cells, iPS cells could be differentiated into adipocytes and that this adipocyte differentiation could be markedly facilitated by transient *PPARγ* gene transduction using an Ad vector.

Because Ad vector-mediated functional gene transduction was found to be effective to increase the differentiation efficiency from ES and iPS cells, we expected that other functional cells could be efficiently differentiated from ES and iPS cells by using an Ad vector. To confirm this finding, both types of cells were differentiated into osteoblasts by Ad vector-mediated transduction of a *Runx2* gene, which was previously proven to be indispensable for osteoblast differentiation [32, 33]. ES and iPS cells were transduced in triplicate with Ad-CA-LacZ or Ad-CA-Runx2 and were cultured with osteogenic supplements. We initially examined activity of ALP, an early osteoblast differentiation marker, in both types of cells, and showed that Ad-CA-Runx2-transduced cells exhibited higher ALP activity than nontransduced or Ad-CA-LacZ-transduced cells (Fig. 5A). These results indicated that early osteoblast differentiation was promoted by Ad vector-mediated *Runx2* gene transfer. Next, to estimate the mature osteoblast differentiation, matrix mineralization in the cells was detected by von Kossa staining. Consistent with the previous report [34], treatment with osteogenic supplements resulted in matrix mineralization in both types of cells, whereas in the absence of additives no calcification was observed (Fig. 5B). We also found that osteoblast differentiation from both ES and iPS cells could be dramatically promoted by Ad vector-mediated *Runx2* gene transduction (Fig. 5B). The level of calcium in Ad-CA-Runx2-transduced ES or iPS cells was approximately eightfold higher than that of nontransduced or Ad-CA-LacZ-transduced cells (Fig. 5C). Semiquantitative RT-PCR analysis also showed that the expression levels of Runx2, osterix, bone sialoprotein, osteocalcin, and type I collagen mRNA were up-regulated in the cells transduced with Ad-CA-Runx2 (Fig. 5D). These results demonstrated that the osteogenic potential in iPS cells was equal to that in ES cells and that efficient osteoblast differentiation from ES and iPS cells could be achieved by exogenous Runx2 expression using optimized Ad vectors.

Discussion

The establishment of an efficient gene transfer system for pluripotent cells would be quite useful for the application of these cells to regenerative medicine. We have previously developed suitable Ad vectors for transducing an exogenous gene into mouse ES cells and ES-EBs and showed that these Ad vectors could be successfully applied to regenerative medicine and basic studies [16, 17]. The aim of this study was to characterize the efficiency of transduction with Ad vectors in mouse iPS cells and to develop efficient methods for inducing the differentiation of mouse iPS cells by means of Ad vector transduction. This is the first study to report the detailed transduction properties of various types of Ad vectors in mouse iPS cells.

We optimized the transduction activity in mouse iPS cells and iPS-EBs by comparing four types of promoters (RSV, CMV, CA, and EF-1α) using Ad vectors. Because iPS cells have been shown to possess mostly the same properties as ES cells [4–6] and the CA and the EF-1α promoter exhibited strong transduction activity in mouse ES cells [16], we speculated that the same results might be obtained in mouse iPS cells. As we expected, mouse iPS cells and iPS-EBs were capable of being efficiently transduced by using a conventional Ad vector containing the CA (and the EF-1α) promoter (Figs. 2A, 3, supporting information Figs. S1, S2, S4). We found that a primary Ad receptor, CAR, was highly expressed in iPS cells (Fig. 1), which were generated from MEFs [4],