Intracellular cytokine staining assay

IFN-γ-secreting $CD8^+$ T cells were detected by the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus kit, PharMingen, San Diego, CA, USA). In brief, lymphocytes were isolated from the mouse spleen. A single cell suspension was incubated with 10 μg/ml of the HIV V3 peptide (NNTRKRIQRGPGRAFVTIGKIGN) for 24 h at 37°C. At 2 h before the end of incubation, 1 μg/ml of GolgiPlug was added. The cells were washed with staining buffer (3% fetal calf serum (FCS), 0.1% sodium azide (NaN_3) in PBS), blocked with 4% normal mouse sera, and stained with phycoerythrin (PE)-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen). The cells were then suspended in 250 μl of Cytofix/Cytoperm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anti-mouse IFN-γ Ab conjugated with fluorescein isothiocyanate (FITC) (PharMingen) at 4°C for 30 min, followed by flow cytometric analysis.

Tetramer assay

The tetramer assay used a PE-conjugated H-$2D^d$/p18 tetramer (RGPGRAFVTI), as previously described.[28] In brief, splenocytes were isolated from mice and incubated for 30 min at 4°C with 4% normal mouse serum in PBS. Cells were stained with FITC-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen) for 30 min at 4°C. After washing twice with the staining buffer (3% FCS, 0.1% NaN_3 in PBS), the cells were incubated with the tetramer reagent for 15 min at 37°C, followed by flow cytometric analysis (Becton Dickinson).

Recombinant vaccinia virus used for the challenge study

Using vPE16 vaccinia virus, the virus challenge experiment was performed as described previously.[28] Vaccinated female mice were intraperitoneally challenged with 10^8 PFU of vaccinia virus vPE16 at 2 or 7 weeks after the final immunization. At 6 days after challenge, the mice were killed, their ovaries were sonicated, and the vPE16 titer was determined by serial 10-fold dilution on a plate of CV1 cells. Infected cells were detected by staining with crystal violet and plaques were counted at each dilution.

Detection of HIV-1-specific Ab

The HIV-1-specific Ab was detected by the Western blotting method and the enzyme-linked immunosorbent assay (ELISA). By Western blotting method, the HIV envelope glycoprotein gp160-coated membrane from the New Lav Blot 1 kit (Bio-Rad, Marnes-la-Coquette, France) was incubated with a 100-fold dilution of mouse serum followed by an affinity-purified horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (ICN Pharmaceuticals Inc., OH, USA). HIV gp160 protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech).

ELISA was performed as described elsewhere.[10] To summarize, 96-well microtiter plates were coated with 10 μg/ml of HIV_{IIIB} gp120 protein (donated by AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They were then treated with 100 μl of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified HRP-labeled anti-mouse Ab or anti-monkey Ab (both from Sigma). The mean Ab titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 s.d.

The HIV-specific neutralizing titer of immune mice or monkeys was also measured. The serially diluted antisera were incubated with 200–300 blue spot-forming units (BFU) of HIV-1_{LAI} at 37°C for 1 h. The mixture was incubated with confluent MAGIC5 cells (from Dr Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan),[50,51] Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 0.2 mg/ml of G418 in a 96-well plate at 37°C for 2 days. The cells were fixed with fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min and stained with staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, 0.4 mg/ml X-gal in PBS) at 37°C for 18–24 h. The staining was stopped by removing the staining solution and the cells were washed twice with PBS. The blue spot in each well was counted after the staining, and the neutralizing titer was calculated as (1−(% infection/% infection of control wells)) × 100. The 50% neutralization dose (ND_{50}) is defined as the concentration of the Ab that reduced the number of infected cells by 50%. The detecting limitation of the assay was 100 ND_{50}/ml.

ELISPOT assay

The frequency of HIV-specific IFN-γ-secreting cells in monkeys was determined using an ELISPOT assay kit (U-Cytech, Utrech, The Netherlands) according to the manufacturer's manual. In brief, 2×10^5 monkey PBMCs were stimulated in triplicate wells with 1 μg/ml of the HIV_{IIIB} gp120 protein for 16 h at 37°C. Nonstimulated cells were used to assess the background. The cells were transferred to an anti-IFN-γ Ab-coated 96-well plate and incubated for 5 h at 37°C. The cells were removed and 200 μl/well of ice-cold deionized water was added to lyse the remaining PBMCs. Subsequently, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with biotinylated anti-IFN-γ Ab for 1 h at 37°C. After 10 washings with PBS-T, 50 μl of gold-labeled anti-biotin Ab was added and incubated for 1 h at 37°C. The plate was washed 10 times with PBS-T, and 30 ml of activator solutions was added. The plate was incubated in the dark for 30 min at room temperature to develop spot formations. After 30 min incubation, the plate was washed with deionized water and air-dried; spots were counted by a computer-assisted video image analysis. The results were expressed as spot-forming cells (SFC) per million cells.

Ad-specific neutralizing assay

Ad5-Luc or Ad5/35-Luc vector (10^7 vp) was incubated with an equal volume of serially diluted normal human sera (anti-Ad5 neutralizing titer <1:4), human antisera (anti-Ad5 neutralizing titer = 1:64), or monkey sera (at weeks 0, 2, 8, and 12 after immunization with Ad5/35-HIV vector) at 37°C for 2 h. The mixture was incubated with confluent Vero cells in a 96-well plate at 37°C for an additional 48 h. The luciferase activity was detected by Luciferase Assay Systems (Promega, Madison, WI, USA).

The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background.

Data analysis

All values were expressed as means $\pm$ standard error (s.e.). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at $P<0.05$ in the statistical analysis.

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ARTICLE

Efficient Gene Transfer into Mouse Embryonic Stem Cells with Adenovirus Vectors

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Efficient and transient gene transfer into embryonic stem (ES) cells is expected to be of use for basic studies in developmental biology and for applications in regenerative medicine. Here, we report the development of an adenovirus (Ad) vector that efficiently expresses foreign genes in mouse ES (mES) cells. We prepared four LacZ-expressing Ad vectors, each of which contained one of the following: Rous sarcoma virus (RSV), cytomegalovirus (CMV), β-actin promoter/CMV enhancer (CA), or EF-1α promoter. While the RSV and CMV promoters were inactive in mES cells, the CA and EF-1α promoters strongly drove LacZ expression in more than 90% of the mES cells. The EF-1α promoter was found to be slightly more efficient than the CA promoter. mES cells were found to express the Ad primary receptor, coxsackievirus and adenovirus receptor, suggesting that while Ad vectors could introduce the exogenous gene into mES cells, the choice of a suitable promoter was critical for efficient gene expression. Fiber-mutant Ad vectors containing RGD or polylysine peptide on the fiber knob mediated efficient LacZ expression, not only in mES cells, but also in feeder cells. Exogenous expression of Oct-3/4 or the dominant-negative mutant of STAT3 (STAT3F) by conventional Ad vectors containing the EF-1α promoter promoted the differentiation of mES cells into the cells of three germ layers, and STAT3F-mediated differentiation was rescued by the coexpression of Nanog. These results suggest that Ad vectors can be used for basic research using ES cells and that they may be of great utility for therapeutic applications in gene-modified regenerative medicine based on ES cells.

Key Words: adenovirus vector, embryonic stem cells, gene therapy, regenerative medicine

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of the developing blastocyst and they give rise to all cell types found in adult organisms [1–3]. ES cells could serve as a renewable source of transplantable tissue-specific stem cells [4–6]. However, despite the importance of ES cells in developmental biology and their potential impact on regenerative medicine, little is known about the signaling pathways in ES cells that govern their unique properties, such as their self-renewal ability and pluripotency. In mouse ES (mES) cells, leukemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (STAT3) is the only well-characterized signaling pathway for self-renewal [7,8]. However, LIF is not necessary in human ES cells, nor in several mES cell lines, suggesting that other signaling pathways are responsible for ES cell self-renewal [9]. The lack of techniques for the genetic manipulation of ES cells may account for the present limits to their usefulness.

Electroporation methods [10], retroviral vectors [11,12], lentiviral vectors [13–15], and a supertransfection method based on a replication system using the polyoma replication origin and large T antigen [16] have been used for exogenous gene expression in ES cells, although lentiviral vectors have been shown to be ineffective at expressing exogenous genes in mouse ES cells, but not in human or simian ES cells [13,15]. In the electroporation and supertransfection methods, gene-modified stable cells are selected using drug-resistance genes, whereas in case of the retrovirus and lentivirus vectors, foreign genes

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are introduced into the chromosome. Therefore, all of these methods mediate long-term constitutive gene expression. However, a long-term gene expression system may be problematic if used in therapeutic applications, as the gene in such a system is continuously expressed, even after cell differentiation. Therefore, efficient vector systems for transient expression are still needed. By using such systems, ES cells could be transfected with a gene that is critical for their differentiation, such as HoxB4, which has been implicated in the self-renewal of definitive hematopoietic stem cells (HSCs) [17].

Adenovirus (Ad) vectors have been extensively used to deliver foreign genes to a variety of cell types *in vitro* and *in vivo* [18,19]. Ad type 5 (Ad5) requires coxsackievirus and adenovirus receptor (CAR) on the cell surface as a primary receptor for infection [20], and its genome persists episomally in host cells [18,19]. Ad vectors show transient gene expression, which is preferable to constitutive expression in certain cases, especially those associated with therapeutic use. Despite the significance of Ad vectors in basic research and gene therapy, little information is available on Ad-vector-mediated gene transfer into ES cells. Here, we describe the construction of suitable Ad vectors for expressing foreign genes in mES cells. To confirm that the Ad vectors were applicable to basic research, we tested the possibility of controlling cell differentiation using three different genes: Oct-3/4, which is implicated in ES cell pluripotency [21]; *NANOG*, which is also critical for ES cell self-renewal [22,23]; and *STAT3F*, which is a dominant-negative mutant of *STAT3* and is able to inhibit LIF/STAT3 signaling [24].

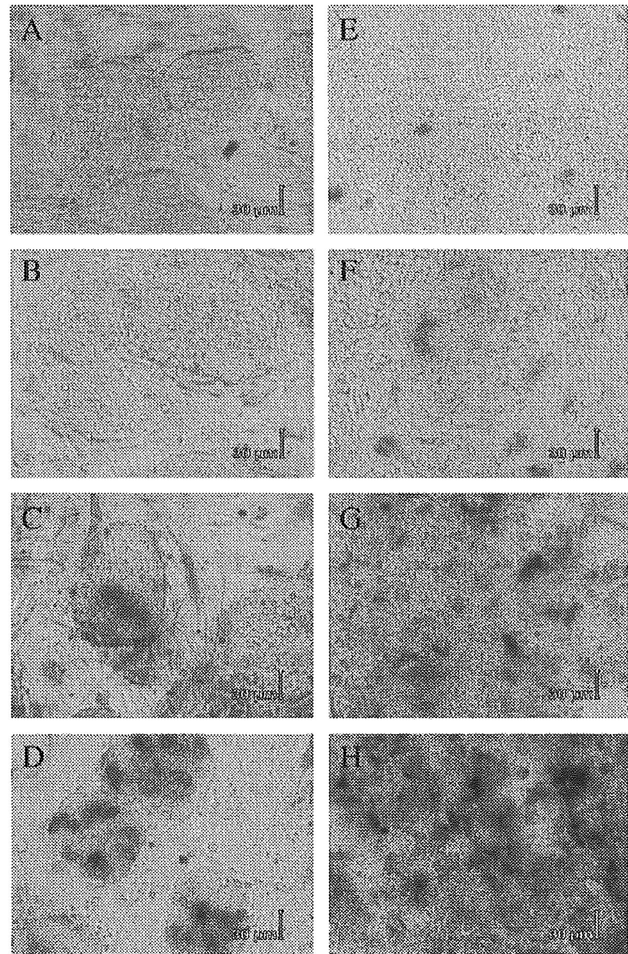

FIG. 1. X-gal staining of Ad vector-transduced mES cells. mES cells (A–D) on feeder cells or (E–H) on gelatin-coated dishes were transduced with 3000 VP/cell Ad-RSV-LacZ (A, E), Ad-CMV-LacZ (B, F), Ad-CA-LacZ (C, G), or Ad-EF-LacZ (D, H) for 1.5 h. Two days after infection, X-gal staining was performed as described under Materials and Methods. Similar results were obtained in three independent experiments.

RESULTS

LacZ Expression in mES Cells Transduced by Ad Vectors

To develop suitable Ad vectors for mES cells, we prepared four LacZ-expressing Ad vectors containing the Rous sarcoma virus (RSV) promoter, cytomegalovirus (CMV) promoter, β-actin promoter/CMV enhancer (CA), or EF-1α promoter (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, and Ad-EF-LacZ, respectively). We infected mES cells cultured on feeder cells with each Ad vector (3000 vector particles (VP)/cell) and examined LacZ expression by X-gal staining (Figs. 1A–1D). Ad-RSV-LacZ- and Ad-CMV-LacZ-infected mES cells showed only low levels of expression of LacZ, but Ad-CA-LacZ- and Ad-EF-LacZ-infected cells expressed high levels of LacZ (Figs. 1A–1D). Although Ad-CA-LacZ mediated LacZ expression in both mES cells and their feeder cells, Ad-EF-LacZ mediated LacZ expression specifically in mES cells and not in feeder cells (Figs. 1C and 1D). We also examined the gene expression levels in mES cells cultured on a feeder-free system, which involved culturing the cells on gelatin-coated dishes (Figs. 1E–1H). The mES cells transduced with Ad-CA-LacZ and Ad-EF-LacZ also efficiently expressed LacZ, whereas the cells transduced with Ad-RSV-LacZ and Ad-CMV-LacZ showed only slight LacZ expression (Figs. 1E–1H). A direct count of the cell number revealed that more than 90% of the cells were transduced with 3000 VP/cell Ad-EF-LacZ, i.e., a rate slightly higher than that observed in the case of Ad-CA-LacZ (data not shown). These results indicate that mES cells efficiently expressed LacZ by Ad vectors when the EF-1α (or CA) promoter was employed to drive the transgene.

Next, we examined the expression of LacZ in mES cells transduced with a different dose of Ad-EF-LacZ. We performed both a luminescence assay (Fig. 2A) and X-gal staining (Fig. 2B) to monitor the dose dependency of Ad-EF-LacZ. The expression of LacZ in mES cells was dose-dependent, and approximately 100% of the cells expressed LacZ at 10,000 VP/cell (Fig. 2). We also investigated the time course of gene expression in mES cells. We transduced mES cells with 3000 VP/cell Ad-EF-

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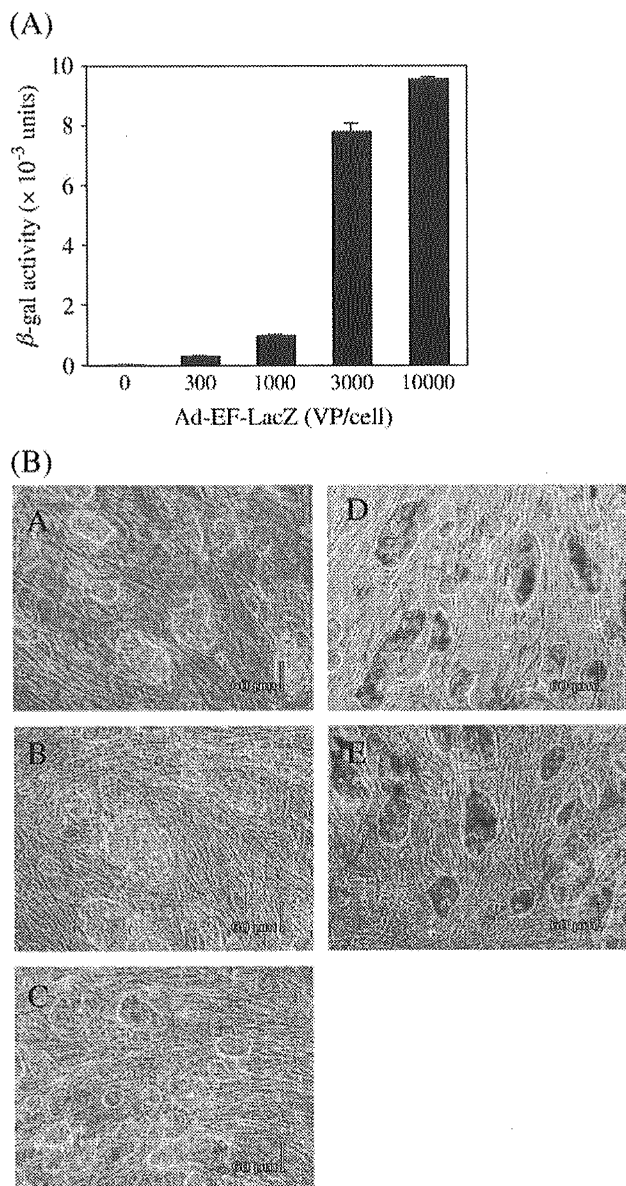

FIG. 2. Dose-dependent LacZ expression in mES cells transduced with Ad-EF-LacZ. mES cells were infected with different amounts of Ad-EF-LacZ for 1.5 h. Two days after infection, LacZ expression was measured by (A) a luminescence assay and (B) X-gal staining as described under Materials and Methods. (B) A, 0; B, 300; C, 1000; D, 3000; E, 10,000 VP/cell. Similar results were obtained in three independent experiments.

LacZ and performed X-gal staining on days 2, 4, 6, 8, and 12. Since the growth of mES cells was rapid and the cells reached confluence, the number of transduced cells was reduced by 1/3 of the original number by passage on day 6. Ad-EF-LacZ mediated LacZ expression transiently, and by day 12, no more LacZ-positive cells were detected (Fig. 3).

To investigate the reasons for which the Ad vectors containing the EF-1α or CA promoter were able to mediate efficient gene expression in mES cells, we examined the expression of CAR, a primary Ad receptor, in mES cells by RT-PCR and Western blot analysis (Fig. 4). We found that mES cells, but not feeder cells, expressed CAR, suggesting that the Ad-vector-mediated efficient gene expression in mES cells was due to the presence of sufficient expression levels of CAR. The expression of Oct-3/4 was examined as a specific marker of undifferentiated ES cells (Fig. 4A).

Next, we examined whether an increase in the efficiency of LacZ expression could be obtained in mES cells cultured in a feeder cell system (Figs. 5A–5D) or in a feeder-free system (Figs. 5E–5H) by using fiber-modified Ad vectors. We constructed three types of fiber-modified Ad vectors containing the EF-1α promoter-driven LacZ cassette: AdRGD-EF-LacZ, which contains RGD peptide in the HI loop of the fiber knob [25]; AdK7-EF-LacZ, which contains a stretch of lysine residues (K7 (KKKKKKK) peptide) in the C terminal of the fiber knob [26]; and AdF35-EF-LacZ, which replaces the fiber shaft and knob domains of Ad5 with those of Ad35 [27]. These Ad vectors infect cells via αv integrin, heparan sulfates, and CD46, respectively [26,28,29]. Among the four types of vectors used, including a conventional vector, Ad-EF-LacZ

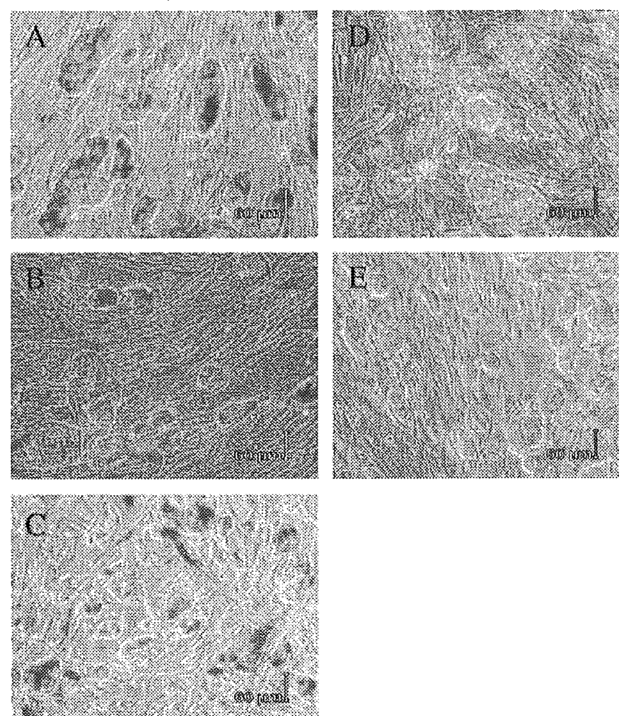

FIG. 3. Time course of LacZ expression in mES cells transduced with Ad-EF-LacZ. mES cells were infected with 3000 VP/cell Ad-EF-LacZ for 1.5 h (day 0). On days (A) 2, (B) 4, (C) 6, (D) 8, and (E) 12, X-gal staining was performed as described under Materials and Methods. On day 6, the number of mES cells was reduced by passage to 1/3 of the original value to avoid overconfluence. Similar results were obtained in three independent experiments.

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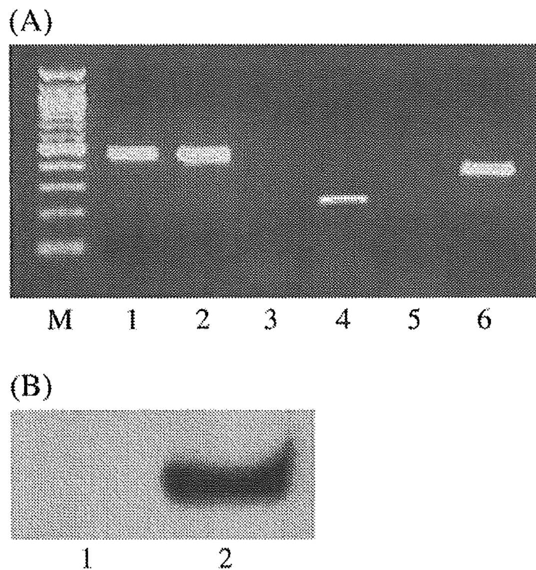

FIG. 4. CAR expression in mES cells. (A) Total RNA from feeder cells (lanes 1, 3, 5) or mES cells on feeder cells (lanes 2, 4, 6) was purified and reverse-transcribed into cDNA, and then the cDNA was amplified by PCR to analyze the expression of G3PDH (lanes 1, 2), CAR (lanes 3, 4), and Oct-3/4 (lanes 5, 6). M represents the molecular marker. (B) Cell lysates (20 μg/lane) from feeder cells (lane 1) or mES cells on feeder cells (lane 2) were loaded onto a 12.5% SDS–polyacrylamide gel, electrophoresed, and subsequently transferred to a membrane. The immunoblot was probed with a polyclonal antibody for mouse CAR.

showed the most efficient and specific expression of LacZ in mES cells (Figs. 5A–5D). While AdF35-EF-LacZ induced only slight LacZ expression in both mES cells and feeder cells, AdRGD-EF-LacZ and AdK7-EF-LacZ mediated LacZ expression in feeder cells rather than mES cells (Figs. 5A–5D). AdRGD-EF-LacZ and AdK7-EF-LacZ are known to infect cells via CAR and via αv integrin and heparan sulfates, respectively. Here, lower levels of LacZ expression were observed in mES cells transduced by AdRGD-EF-LacZ and AdK7-EF-LacZ than in those transduced by Ad-EF-LacZ; these results were most likely due to the sequestration of AdRGD-EF-LacZ and AdK7-EF-LacZ by the feeder cells. In the feeder-free system, Ad-EF-LacZ, AdRGD-EF-LacZ, and AdK7-EF-LacZ expressed comparable levels of LacZ (Figs. 5E–5H). Taken together, these results demonstrate that the conventional Ad vector containing the EF-1α promoter is a suitable vector for mES cells, when using either a feeder cell system or a feeder-free system, and that fiber-modified Ad vectors with RGD or polylysine peptides mediate gene expression in both mES cells and feeder cells.

Regulation of Cell Differentiation by Ad-Vector-Mediated Transduction

To confirm that the Ad vectors were applicable to basic research, we introduced functional genes that regulate cellular differentiation into mES cells. We constructed three vectors, Ad-EF-Oct3/4, Ad-EF-Nanog, and Ad-EF-STAT3F, which expressed Oct-3/4, NANOG, and a dominant-negative mutant of STAT3, respectively. Oct-3/4 is highly expressed in ES cells and has been shown to be essential for maintaining pluripotency in mES cells [21]. However, overexpression of Oct-3/4 in mES cells is known to promote differentiation [21]. mES cells infected with Ad-EF-Oct3/4 proceeded to a moderate level of differentiation (Fig. 6C). In STAT3F, the tyrosine residue at amino acid position 705 in STAT3 is mutated to phenylalanine. Phosphorylation of Tyr705 is required for dimerization and nuclear translocation. When expressed at high levels, STAT3F has been shown to block the activation of endogenous STAT3 in various cell

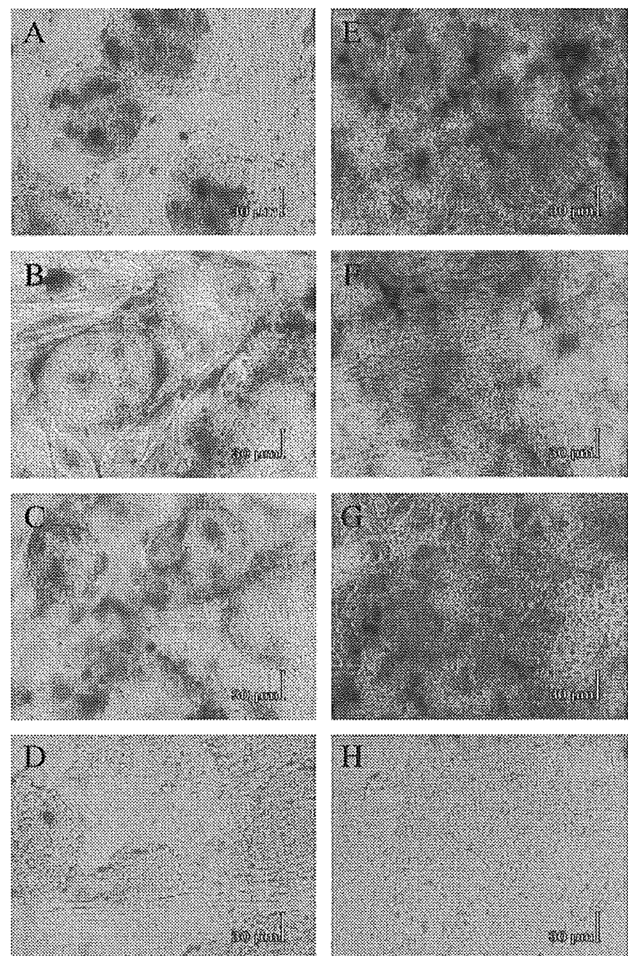

FIG. 5. X-gal staining of mES cells transduced with fiber-modified Ad vectors. mES cells (A–D) on feeder cells or (E–H) on gelatin-coated dishes were transduced with 3000 VP/cell Ad-EF-LacZ (A, E; the images correspond to those in Fig. 1), AdRGD-EF-LacZ (B, F), AdK7-EF-LacZ (C, G), or AdF35-EF-LacZ (D, H) for 1.5 h. Two days after infection, X-gal staining was performed as described under Materials and Methods. Similar results were obtained in three independent experiments.

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types [24]. When we infected mES cells with Ad-EF-STAT3F, about half of the cells died, and the surviving cells proceeded to full differentiation (Fig. 6D). This finding indicates that LIF/STAT3 signaling, which is the only well-known pathway for the self-renewal of mES cells, was inhibited by STAT3F. Furthermore, this phenotype was partially rescued by co-infection of Ad-EF-Nanog, indicating that NANOG, which is also known to be a pluripotency-sustaining factor [22,23], maintained the survival and self-renewal of mES cells independent of LIF/STAT3 signaling (Figs. 6E and 6F). We also confirmed the differentiation of mES cells transduced with Ad-EF-Oct3/4 and Ad-EF-STAT3F by RT-PCR analysis of each germ-layer marker gene (Fig. 7). LacZ- and Nanog-transduced mES cells were undifferentiated and expressed the ES-cell-specific markers Oct-3/4 and NANOG. When Oct-3/4 was transduced into the mES cells, NANOG expression was no longer detected and, in turn, the three germ-layer marker genes FGF5 (primitive ectoderm), Brachyury (T) (mesoderm), and GATA6 (endoderm) [30] were expressed. mES cells transduced with Ad-EF-STAT3F also expressed these differentiation markers. Co-infection of Ad-EF-STAT3F with Ad-EF-Nanog resulted in expression patterns similar to those obtained with Ad-EF-LacZ; moreover, the cells were not found to express the differentiation markers, thus suggesting that NANOG was capable of maintaining the self-renewal of mES cells—even in the absence of LIF/STAT3 signaling—by Ad-vector-mediated gene transfer.

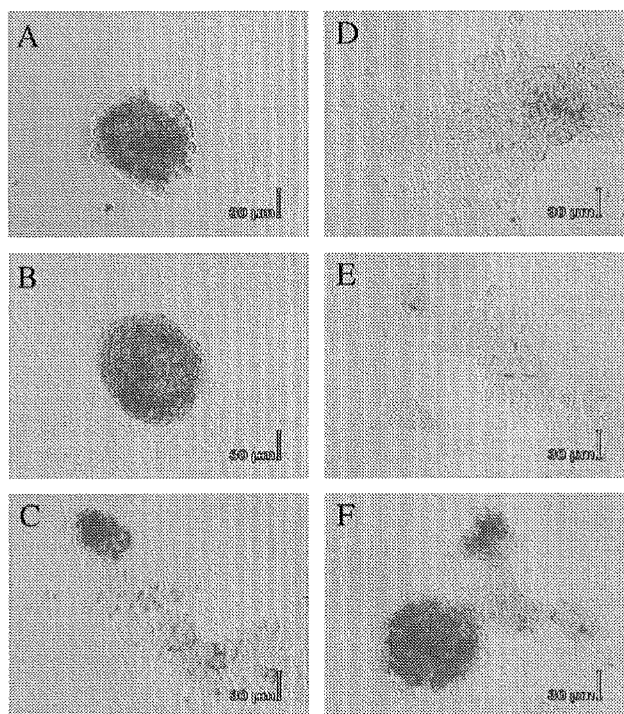

FIG. 6. Introduction of functional genes into mES cells by Ad vectors containing EF-1α promoter. mES cells (1×10^4 cells) were seeded, and on the following day, the cells were transduced with 3000 VP/cell of (A) no vector, (B) Ad-EF-LacZ, (C) Ad-EF-Oct3/4, or (D) Ad-EF-STAT3F for 1.5 h. mES cells were also co-infected with 3000 VP/cell Ad-EF-STAT3F and 3000 VP/cell of (E) Ad-EF-LacZ or (F) Ad-EF-Nanog for 1.5 h. On day 3, each cell was reinfected by the same vectors. On day 5, alkaline phosphatase staining was performed as described under Materials and Methods. Alkaline phosphatase-positive red cells indicate undifferentiated ES cells. Similar results were obtained in three independent experiments.

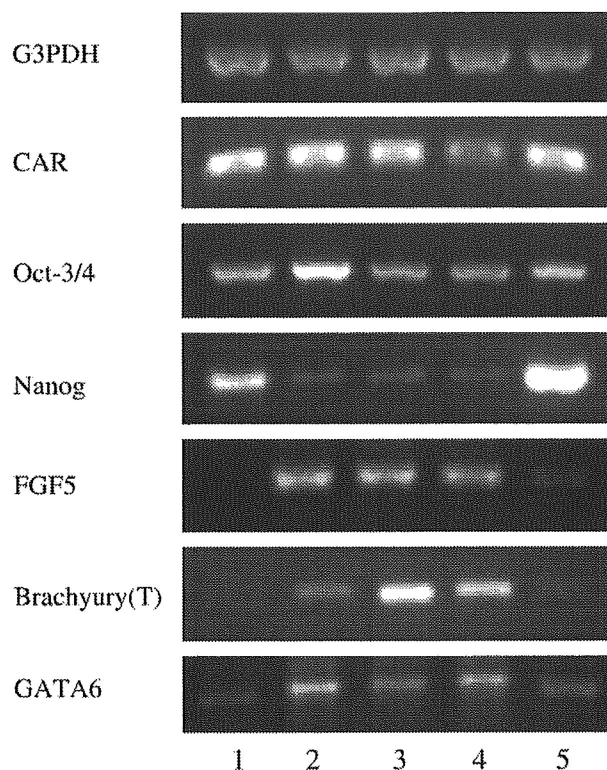

FIG. 7. RT-PCR analysis of germ-layer markers. Total RNA was isolated from mES cells transduced with Ad-EF-LacZ (lane 1), Ad-EF-Oct3/4 (lane 2), Ad-EF-STAT3F (lane 3), Ad-EF-STAT3F and Ad-EF-LacZ (lane 4), or Ad-EF-STAT3F and Ad-EF-Nanog (lane 5) as described in the legend to Fig. 4. RT-PCR analysis was performed as described under Materials and Methods.

DISCUSSION

In this study, we successfully constructed Ad vectors for ES cells and showed that these optimized vectors were applicable for use in experiments in the field of ES cell biology. Psarras and colleagues recently reported that Ad vectors preferentially transduced feeder cells, but not ES cells [31]. They used an Ad vector containing the CMV promoter and suggested that ES cells might not express CAR. However, we demonstrated that mES cells did express CAR and these cells also showed high levels of LacZ expression mediated by an Ad vector containing the EF-1α promoter (Figs. 1 and 4). We also demonstrated that the CMV promoter was not appropriate for Ad-vector-

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mediated transduction into ES cell. Chung and colleagues reported that in a transient expression system using a cationic liposome/plasmid complex, the EF-1α and CA promoters were highly active in mouse ES cells, while the CMV promoter was inactive [32]. Our present results obtained using Ad vectors were consistent with those previous findings. Moreover, in the case of conventional fiber-unmodified Ad vectors, we found that while Ad vectors containing the CA promoter mediated LacZ expression in both mES cells and their feeder cells, Ad vectors containing the EF-1α promoter mediated LacZ expression specifically in mES cells, not in feeder cells, at least at the lower vector concentration. This result was probably due to the relatively weaker activity of the EF-1α promoter of the feeder cells compared to that of the CA promoter, whereas in the mES cells, the relative activity of the EF-1α promoter would be similar to (or slight higher than) that of the CA promoter. These properties led to the induction of only slight levels of LacZ expression in the feeder cells treated with Ad-EF-LacZ. We concluded that EF-1α is the most appropriate promoter for gene transfer into mES cells.

We next constructed various fiber-modified Ad vectors to improve the efficiency of mES cell transduction. In our previous study, fiber-modified Ad vectors containing RGD or K7 peptides were shown to be more efficient than the conventional Ad vector in many kinds of CAR-positive and -negative cells [25–27]. However, the conventional Ad vector showed highly efficient and specific transduction into mES cells, whereas fiber-modified vectors such as AdRGD-EF-LacZ and AdK7-EF-LacZ preferentially transduced feeder cells (Figs. 1 and 5). Although we used E14 mES cells in the present study, another mouse CMTI-1 ES cell line was also efficiently transduced with the conventional Ad vector containing EF-1α promoter (data not shown). CAR was found to be expressed in mES cells, but not in feeder cells (Fig. 4). These findings indicate that the conventional vector is much easier to use for the infection of mES cells than are fiber-modified vectors. To date, it remains unknown whether CAR is also expressed in other ES cell lines such as human ES cells. Smith-Arica and colleagues reported that an Ad vector containing the RSV promoter was efficient for the infection of mouse and human ES cells [33], suggesting that CAR might be expressed not only in mouse ES cells, but also in human ES cells. Because the EF-1α promoter was found to be more active than the RSV promoter in ES cells (Fig. 1), the Ad vector containing the EF-1α promoter could be a rewarding vehicle for gene transfer into many types of ES cells.

Therefore, to determine the usefulness of this vector for basic research, functional genes were introduced into mES cells. It is well known that STAT3 activation by LIF is essential for LIF-mediated ES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation [7]. Ad-EF-STAT3F strongly promoted mES cells to apoptosis or differentiation into three germ layers without exhibiting any nonspecific toxicity. NANOG is a recently identified transcription factor that is expressed specifically in ES cells and the inner cell mass of blastocysts [22,23]. It was also found that NANOG maintains the pluripotency of ES cells independent of LIF/STAT3 signaling. Co-infection experiments of Ad-EF-STAT3F with Ad-EF-Nanog into mES cells showed that the differentiation-suppressing ability of NANOG negated the differentiation-promoting function of STAT3F and thus led to the continued support of the ES cell self-renewal, suggesting that the two proteins were efficiently expressed in mES cells by the Ad vectors. Oct-3/4 is a POU family transcription factor and a marker for ES cells. However, its overexpression results in differentiation into three germ layers via unidentified mechanisms [21]. Ad-EF-Oct3/4 also induced mES cells to differentiate, although the morphology of the cells in the case of Ad-EF-Oct3/4 infection differed from that in the case of Ad-EF-STAT3F infection. These results lead to the expectation that Ad vectors containing the EF-1α promoter will be useful for studies in the field of ES cell biology.

To date, almost all vectors for ES cells (e.g., retroviral vectors or lentiviral vectors) are applied for long-term constitutive expression, although in some cases, transient gene expression is required instead. For example, when ES cells are transiently transduced with HoxB4, a homeotic selector gene implicated in the self-renewal of definitive HSCs, and when they are subsequently cultured on OP9 stroma cells, they differentiate into HSCs and can be expanded *in vitro* [17]. However, it is known that the constitutive retroviral expression of HoxB4 in ES cells prevents HSCs from expanding in culture, which is in turn reflective of its undesirable effects on hematopoietic differentiation. To obtain purified HSCs, a complex system for inducible HoxB4 expression has been developed to control the period of HoxB4 expression [17]. In cases such as this, Ad vectors could be useful, as their expression is transient.

In the present study, we developed an efficient Ad vector system for ES cells and demonstrated the potential usefulness of this vector for basic research. In addition, this system might also have therapeutic applications in the field of gene-modified regenerative medicine based on ES cells.

MATERIALS AND METHODS

ES cell cultures. Mitotically inactivated mouse embryonic fibroblasts (MEFs) and LIF-containing ES cell culture medium were purchased from Specialty Media, Inc. (Phillipsburg, NJ, USA). Mouse E14 ES cells were kindly provided by N. Yoshida (University of Tokyo, Tokyo, Japan). mES cells were routinely passaged every 3–5 days on MEF layers after trypsinization. In feeder-free culture, mES cells on MEFs were trypsinized and MEF layers were separated with mES cells by culturing at 37°C for 40 min. Floating cells were regarded as ES cells and transferred to gelatin-coated dishes for the feeder-cell-free condition.

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Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method [34,35]. Briefly, pHMCA5 and pHMEF5 were constructed by changing the CMV promoter of pHMCMV5 [35] into the CA promoter (a kind gift from J. Miyazaki, Osaka University, Osaka, Japan) [36] or the EF-1α promoter, which is derived from pEF/myc/nuc (Invitrogen, Carlsbad, CA, USA). The LacZ gene, which is derived from pCMVβ (Marker Gene, Inc., Eugene, OR, USA), was inserted into pHMCMV5, pHMCA5, and pHMEF5, resulting in pHMCMV5-LacZ, pHMCA5-LacZ, and pHMEF5-LacZ, respectively. pHMCMV5-LacZ, pHMCA5-LacZ, and pHMEF5-LacZ were then digested with I-*Ceu*I and PI-*Sce*I and ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 [34], resulting in pAdHM4-CMVLacZ1, pAdHM4-CALacZ1, and pAdHM4-EFLacZ1, respectively. I-*Ceu*I/PI-*Sce*I-digested pHMEF5-LacZ was also ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 [34], pAdHM15-RGD [37], pAdHM41-K7(C) [26], or pAdHM34 [27], resulting in pAdHM4-EFLacZ1, pAdHM15-RGD-EFLacZ1, pAdHM41-K7-EFLacZ1, or pAdHM34-EFLacZ1, respectively.

To generate the viruses, *Pac*I-digested Ad vector plasmids were transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Viruses (Ad-CMV-LacZ, Ad-CA-LacZ, Ad-EF-LacZ, AdRGD-EF-LacZ, AdK7-EF-LacZ, and AdF35-EF-LacZ) were prepared as described previously [34]. Ad-EF-Oct3/4, Ad-EF-Nanog, and Ad-EF-STAT3F were similarly generated by using Oct-3/4 (a kind gift from H. Niwa, RIKEN, Kobe, Japan), NANOG (a kind gift from S. Yamanaka, Nara Institute of Science and Technology, Nara, Japan), or STAT3F (a dominant-negative form of STAT3; a kind gift from S. Akira, Osaka University, Osaka, Japan) cDNA, pHMEF5, and pAdHM4. Ad-RSV-LacZ was obtained from M. A. Kay (Stanford University, CA, USA). The virus was purified with $CsCl_2$ 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 −70°C. The determination of virus particles and the biological titer were accomplished spectrophotometrically by the method of Maizel *et al.* [38] and by using an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA, USA), respectively. The PFU-to-particle ratio was 1:46 for Ad-CMV-LacZ, 1:41 for Ad-RSV-LacZ, 1:22 for Ad-CA-LacZ, 1:44 for Ad-EF-LacZ, 1:17 for AdRGD-EF-LacZ, 1:31 for AdK7-EF-LacZ, 1:44 for AdF35-EF-LacZ, 1:44 for Ad-EF-Oct3/4, 1:35 for Ad-EF-Nanog, and 1:23 for Ad-EF-STAT3F.

LacZ assay. mES cells (1×10^5 cells) were seeded onto a 12-well dish. On the following day, the cells were transduced with 3000 VP/cell or the indicated dose of conventional Ad vectors (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, and Ad-EF-LacZ) and fiber-modified Ad vectors (Ad-EF-LacZ, AdRGD-EF-LacZ, AdK7-EF-LacZ, AdF35-EF-LacZ) for 1.5 h. After 2, 4, 6, 8, or 12 days in culture, the cells were washed with PBS, fixed with 0.5% glutaraldehyde, and stained with X-gal solution (1.3 mM $MgCl_2$, 15 mM NaCl, 44 mM Hepes, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 0.05% X-gal (Nippon Gene, Toyama, Japan) solution dissolved in dimethylformamide). β-Gal activity was quantitatively measured with Luminescent β-Gal Kit (Clontech).

Alkaline phosphatase staining. A small number of feeder-free mES cells (1×10^4 cells) were seeded onto a gelatin-coated 12-well dish. On the following day, the cells were transduced with 3000 VP/cell of Ad-EF-LacZ, Ad-EF-Oct3/4, Ad-EF-Nanog, or Ad-EF-STAT3F. On day 3, each cell type was infected again by the same vectors. On day 5, alkaline phosphatase staining was performed with an ES Cell Characterization Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions.

Rt-PCR analysis. Feeder-free mES cells were infected with Ad-EF-LacZ, Ad-EF-Oct3/4, Ad-EF-STAT3F, or Ad-EF-Nanog for 1.5 h at 3000 VP/cell. At 4 days after infection, total RNA was isolated from the cells by Isogen reagent (Nippon Gene). RNA was reverse-transcribed using a SuperScript First-Strand Synthesis System (Invitrogen). Synthesized cDNA was amplified by PCR using ExTaq DNA polymerase (Takara, Tokyo, Japan). The sequences of the specific primers were as follows: G3PDH(F), 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH(R), 5′-TCCACCACCCTGTTGCTGTA-3′; CAR(F), 5′-TGATCATTTTGTATTCTGGA-3′; CAR(R), 5′-TTAACAAGAACGGTCAGCAG-3′; Oct-3/4(F), 5′-GTTTGCCAAGCTGCTGAAGC-3′; Oct-3/4(R), 5′-TCTAGCCCAAGCTGATTGGC-3′; Nanog(F), 5′-ATGGT CT GATTC AGAAGG GC-3′; Nanog(R), 5′-TTCACCTCCAAATCACTGGC-3′; FGF5(F), 5′-GAAGCGGCTCGGAACATAGC-3′; FGF5(R), 5′-GGACGCATAGGTATTATAGC-3′; Brachyury T(F), 5′-CAGGAGGATGTTCCCGGTGC-3′; Brachyury T(R), 5′-TCCGAGGTTCATACTTATGC-3′; GATA6(F), 5′-GCCAAACTGAGCCCCTTCGC-3′; GATA6(R), 5′-GGGGGGCTGTCGGCGGAGGC-3′. The cycle conditions were 30 s at 94°C, followed by 30 cycles of 5 s at 94°C, 10 s at 60°C, and 1 min at 72°C and a final extension of 7 min at 72°C.

Western blotting. A rabbit polyclonal antibody for mouse CAR was prepared using a peptide (KTQYNQVPSEDFERAPQC). Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 50 mM Tris–Cl (pH 7.4), 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (BD Biosciences, San Jose, CA, USA)). SDS–PAGE was performed under reducing conditions on 12.5% polyacrylamide gels, and the resolved proteins were transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membranes was blocked with 0.1% Tween 20 and Tris-buffered saline containing 2% bovine serum albumin and 3% dry milk at pH 7.4 for 12 h at 4°C. Polyclonal antibody against mouse CAR was added, and the incubation was continued for 3 h. After being washed with 0.1% Tween 20 and Tris-buffered saline, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse antibodies (Cell Signaling Technology, Beverly, MA, USA) for 1.5 h. After extensive washing, the band was visualized by chemiluminescence using an ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA). Signals were read with LAS-3000 (Fujifilm, Tokyo, Japan).

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RESEARCH ARTICLE

Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities

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Adenoviral gene transfer to hematopoietic stem cells (HSCs)/progenitors would provide a new approach to the treatment of hematopoietic diseases and study of the hematopoietic system. We have previously reported that an adenovirus (Ad) vector composed of whole Ad serotype 35 (Ad35), which belongs to subgroup B, shows efficient gene transfer into human bone marrow CD34⁺ cells. However, Ad35 vector-mediated transduction into human HSCs/progenitors has not yet been fully optimized. In the present study, we have systematically examined promoter activity in the context of Ad35 vectors in human bone marrow CD34⁺ cells and primitive CD34⁺ subsets to optimize the transduction efficiency in human hematopoietic stem/progenitor cells. In the first of the transduction experiments, the improved in vitro ligation method was applied to Ad35 vector construction to allow for simple and efficient production of an E1/E3-deleted Ad35 vector. Using this method, we constructed a series of Ad35 vectors encoding the enhanced green fluorescence protein (GFP) under the control of a variety of strong viral and cellular promoters. Of the six types of promoters tested, significantly higher transduction efficiencies were achieved with the human elongation factor 1α promoter (EF1α promoter), the human cytomegalovirus (CMV) immediate-early 1 gene enhancer/β-actin promoter with β-actin intron (CA promoter), and the CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi promoter) in the human CD34⁺ cells and the immature subsets (CD34⁺CD38$^{low/-}$ and CD34⁺AC133⁺ subsets). In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34⁺ cells and the immature hematopoietic subsets. Furthermore, the CA promoter-mediated GFP-expressing cells differentiated into progenitor cells of all lineages. These results indicate the construction of an optimized Ad35 vector backbone for efficient transduction into HSCs/progenitors.
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Introduction

Hematopoietic stem cells (HSCs) have the potential for self-renewal and multilineage differentiation into all mature blood cells. Hence, efficient transduction into HSCs would afford the opportunity to treat a number of diseases that result from abnormal blood cell function, and would be a powerful tool for study of the regulation of proliferation, differentiation, and trafficking of HSCs. For gene transfer into HSCs, moloney-derived retrovirus vectors and lentivirus vectors are often used, although the transduction efficiencies of the retrovirus vectors are disappointingly low in immature HSC/progenitors, probably due to the quiescent state of HSCs and the lack of suitable receptors for vector binding.[1,2] Lentivirus vectors have recently shown promise,[3,4] but their safety remains to be established.

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Among the various types of vectors, adenovirus (Ad) vectors have been widely used for delivery of foreign genes in not only experimental studies but also clinical trials. Advantages which make Ad vectors an attractive vehicle for gene transfer include the ability to easily prepare high-titer stocks of purified vectors, efficient escape from the endosome, and the ability to transport their DNA genome into the nucleus, allowing for efficient transduction in quiescent cells. However, the utility of commonly used Ad vectors, which are based on Ad serotype 2 (Ad2) or Ad serotype 5 (Ad5) belonging to subgroup C, for transduction into human CD34⁺ cells has been limited[5,6] because a primary receptor for Ad2 and Ad5, coxsackievirus and adenovirus receptor (CAR), and second receptors, $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins, are not expressed at sufficient levels in human CD34⁺ cells.[5,7,8] In contrast, it has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34⁺ cells and hematopoietic cell lines.[5,9] We have therefore developed a novel Ad vector, Ad35 vector, which is composed of whole Ad35, and have demonstrated that Ad35 vectors achieve higher levels of transduction efficiency without significant

toxicity in human bone marrow CD34+ cells than both conventional Ad5 vectors and chimeric Ad5F35 vectors, which are fiber-substituted Ad5 vectors containing Ad35 fiber proteins.[10] Ad35 recognizes CD46 (membrane cofactor protein) as a cellular receptor,[11,12] and CD46 is ubiquitously expressed in almost all human cells except for erythrocytes,[13,14] including human cord blood CD34+ cells.[15] Therefore, human CD34+ cells would be considered to be a suitable target for Ad35 vectors.

In addition to receptor expression in target cells, the choice of promoters that drive expression of introduced genes is another crucial determinant for transduction efficiency. Optimization of the promoter leads not only to increased transgene expression but also to decreased vector dose and side effects. A variety of promoters have been used for transduction into human CD34+ cells, including the phosphoglycerate kinase 1 promoter (PGK promoter),[16] the human cytomegalovirus immediate-early region promoter/enhancer (CMV promoter),[17,18] and the CMV immediate-early enhancer/the chicken β-actin promoter with the β-actin intron sequence (CA promoter).[8] However, few studies have simultaneously compared the relative strength of various types of promoters in human CD34+ cells,[19] and information regarding promoter activities in human CD34+ cells is controversial. In addition, the promoter activities have not been fully evaluated in immature CD34+ subpopulations. It is well known that human CD34+ cells are morphologically and functionally heterogeneous and that HSCs/progenitors constitute only a fraction of all CD34+ cells. It is of great importance to evaluate the transduction efficiencies in immature hematopoietic subpopulations.

In the present study, we first applied the improved *in vitro* ligation method developed by Mizuguchi and Kay[20,21] to Ad35 vector construction to facilitate the generation of Ad35 vectors. Second, promoter activities in the context of Ad35 vectors were systematically evaluated in whole human bone marrow CD34+ cells and immature CD34+ subpopulations (CD34+CD38$^{low/-}$ and CD34+AC133+ subsets) to optimize Ad35 vector-mediated transduction into human hematopoietic stem/progenitors. Finally, the proliferative and differentiation potential of the Ad35 vector-mediated transduced CD34+ cells was examined by a colony-forming assay.

Results

Construction of E1/E3-deleted Ad35 vectors by the improved in vitro ligation method

To construct E1/E3-deleted Ad35 vectors simply and efficiently, the improved *in vitro* ligation method was applied to the construction of Ad35 vectors. This method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method by which conventional Ad5 vectors can be constructed. First, all of the E1a region and most of the E1b region were deleted to make the Ad35 vectors replication-incompetent. Then, the three unique restriction sites (I-*Ceu*I, *Swa*I, and PI-*Sce*I) were introduced into the E1 deletion site to efficiently insert foreign genes into the E1 deletion site of the vector plasmid by a single *in vitro* ligation. The I-*Ceu*I and PI-*Sce*I sites were used for insertion of foreign genes, while the *Swa*I site was used to reduce the generation of parental, nonrecombinant plasmid. Next, to increase the packaging capacity of the Ad35 vector genome, most of the E3a and E3b regions were deleted. The resulting vector plasmids, pAdMS2, pAdMS3, and pAdMS4, contain the complete Ad35 genome minus the E1 (pAdMS2) or E3 region (pAdMS3) or E1/E3 region (pAdMS4) (Figure 1). Approximately 3 and 1.9 kb of the E1 and E3 regions, respectively, were deleted in pAdMS4.

The Ad35 vector plasmids containing a green fluorescence protein (GFP) gene under the control of a variety of promoters were produced by *in vitro* ligation of I-*Ceu*I/PI-*Sce*I-digested pAdMS4 and the shuttle plasmids containing a GFP expression cassette. SbfI-linearized Ad35 vector plasmids were transfected into VK10-9 cells (293 cells expressing Ad5 E4 proteins as well as E1 proteins),[22] and the cells were cultured for 10–14 days to produce recombinant Ad35 vectors, followed by the routine method for Ad5 vector preparation. VK10-9 cells can support the replication of Ad35 vectors; however, Ad35 vectors can not grow on 293 cells, as described previously.[10] The final yields of the Ad35 vectors were equivalent to those described previously.[10,23]

Relationship between CD46 expression and transduction efficiency in human bone marrow CD34+ cells

Recently, the complement regulatory protein CD46 has been identified as a cellular receptor for Ad subgroup B.[11,12] To study the role of CD46 in Ad35 vector-mediated transduction into human bone marrow CD34+ cells, we evaluated the relationship between CD46 expression levels and the transduction efficiencies of Ad35 vector containing the CMV promoter. The CMV promoter is generally regarded as one of the most powerful

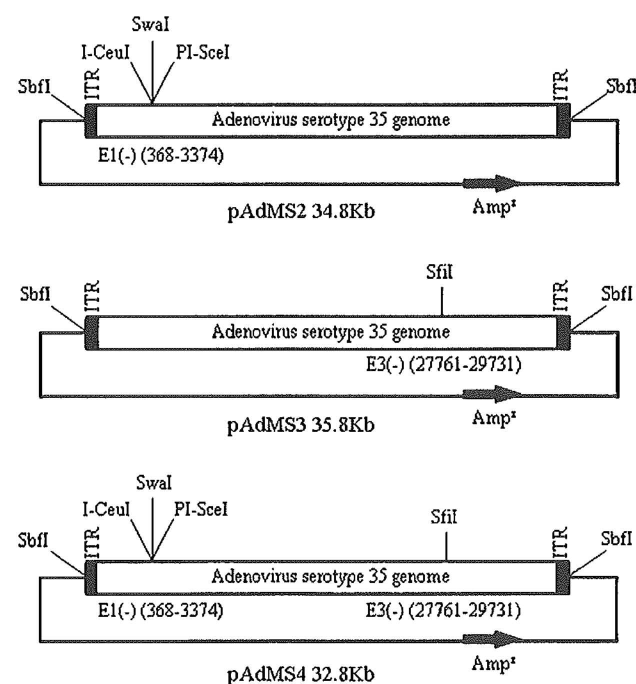

Figure 1 Structure of the vector plasmids pAdMS2, -3, and -4 for construction of Ad35 vectors by the improved in vitro ligation method.

promoters[24] and is widely used in transduction experiments. As shown in Figure 2a, almost all the bone marrow CD34+ cells expressed high levels of CD46, similar to the cord blood CD34+ cells.[15] Significant amounts of CD46 were detected in all the CD34+ cells from three different donors (data not shown). In the transduction experiments using Ad35 vector containing the CMV promoter, the percentage of GFP-positive cells was proportional to the vector concentration at the lower vector doses of 1000–6000 VP/cell (Figure 2b). However, despite the high levels of CD46 expression, the transduction efficiencies of the Ad35 vector did not correlate with the vector concentrations at the higher vector dose reaching a plateau at more than 12 000 VP/cell. There was no correlation between the levels of CD46 expression and the GFP expression levels (Figure 2c). These data indicate that factors other than the CD46 expression levels also determine the transduction efficiencies of Ad35 vectors in human bone marrow CD34+ cells.

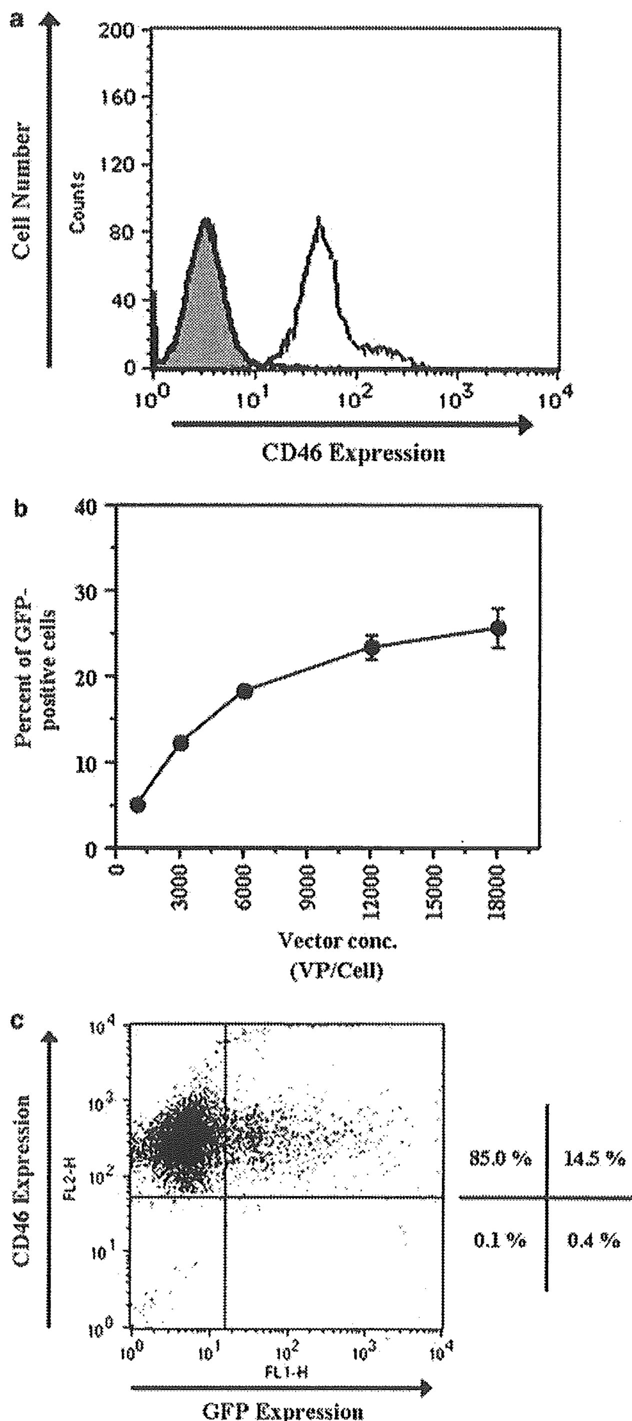

Figure 2 (a) CD46 expression in human bone marrow CD34+ cells. The cells were incubated with FITC-conjugated anti-CD46 antibody. As a negative control, the cells were incubated with an irrelevant antibody (shaded histogram). Similar levels of CD46 were found in the cells from three different donors. (b) Dose–response of the percentage of GFP-positive cells following transduction with Ad35 vector containing a CMV promoter-driven GFP expression cassette. Human bone marrow CD34+ cells were transduced with the Ad35 vector at the indicated vector concentrations for 6 h, washed, and resuspended in medium. After 48 h later, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of three experiments. (c) Relationship between the CD46 expression level on human bone marrow CD34+ cells and GFP expression levels following Ad35 vector transduction. The cells were transduced with the Ad35 vector containing the CMV promoter at 6000 VP/cell for 6 h, washed and resuspended in the medium. After 48 h of incubation and washing, the transduced cells were incubated with an anti-CD46 antibody. The cells were then washed, resuspended, and incubated with PE-conjugated second antibody. The percentage of stained cells found in each quadrant is indicated. Data shown are from one representative experiment of the three performed.

Transduction with Ad35 vectors containing various types of promoters in human bone marrow CD34+ cells

The refractoriness of the human CD34+ cells to Ad35 vector-mediated transduction might be due to promoters that drive expression of a foreign gene. To examine the promoter activities in the CD34+ cells, we investigated the transduction efficiencies of the Ad35 vectors containing various types of promoters in the CD34+ cells at 6000 VP/cell. The following promoters were tested: the CMV promoter, the EF1α promoter, the CA promoter, the mouse PGK promoter, the murine stem cell virus (MSCV) long terminal repeat (LTR) promoter (MSCV promoter), and the CMV promoter/enhancer containing the largest intron of CMV (intron A) (CMVi promoter). Of the six types of promoters, the CA, EF1α, and CMVi promoters were found to allow higher levels of GFP expression than the CMV, PGK, and MSCV promoters (Figure 3). In particular, the highest percentage of GFP-positive cells was obtained with the CA promoter (53.8%). The relative promoter strength in terms of percentage of GFP-positive cells was the CA (53.8%) > CMVi (44.0%) > EF1α (40.9%) > PGK (23.7%) > CMV (20.3%) > MSCV (10.5%). The mean fluorescence intensity (MFI) was also more than 3.5-fold higher with the CA and CMVi promoters than with the other types of promoters. Continuous incubation of the CD34+ cells with Ad35 vector containing the CA promoter at 6000 VP/cell for 48 h led to an increase in GFP-positive cells up to 67% (data not shown). These data indicate that the transduction efficiencies in human bone marrow CD34+ cells are largely dependent on the promoter and that the CA promoter is the most active in CD34+ cells among the six types of promoters examined in the present study.

Next, to determine whether the Ad35 vectors had infected the CD34+ cells that did not express GFP, the

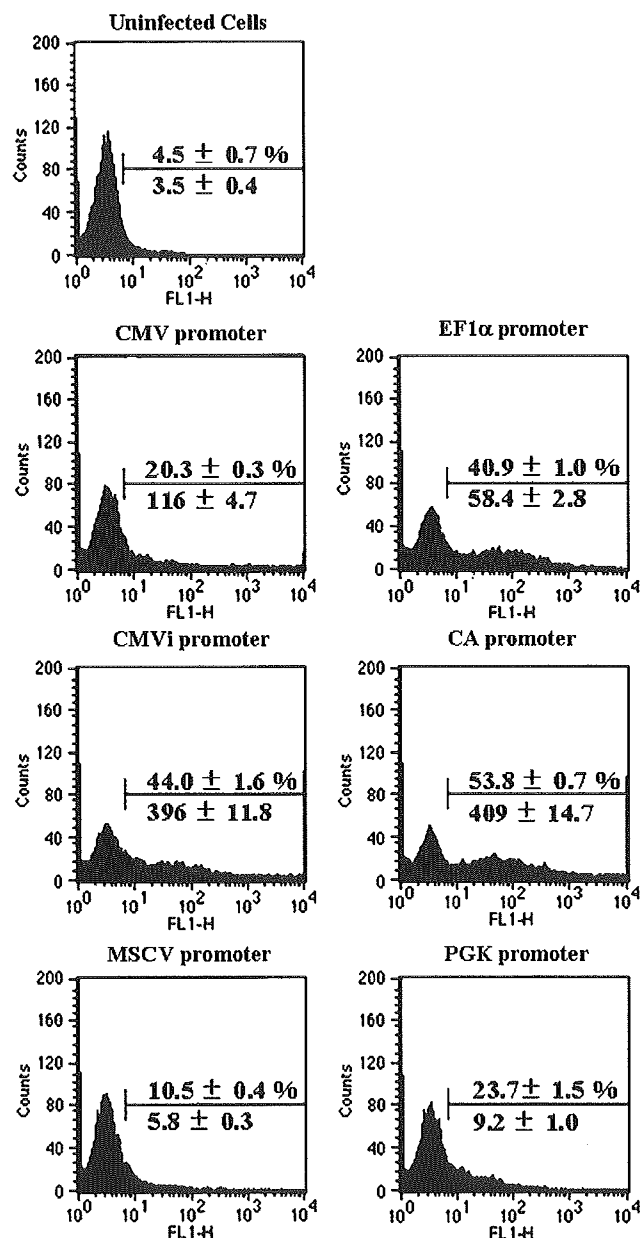

Figure 3 Comparison of promoter activities in human bone marrow CD34+ cells transduced with Ad35 vectors. The results are shown as a percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The CD34+ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of three experiments.

amounts of intracellular vector genomes in the GFP-positive and -negative fractions were measured by real-time PCR. As CD34+ cells are functionally and morphologically heterogeneous, the promoters may not be active in all CD34+ cells. Real-time PCR analysis demonstrated that approximately 300 copies of the Ad35 vector genomes per β-actin copy were detected in both GFP-positive and -negative fractions following transduction with the Ad35 vectors containing any type of promoter (Figure 4). These results suggest that Ad35 vectors would infect all CD34+ cells, probably via CD46; however, not all infected cells express GFP.

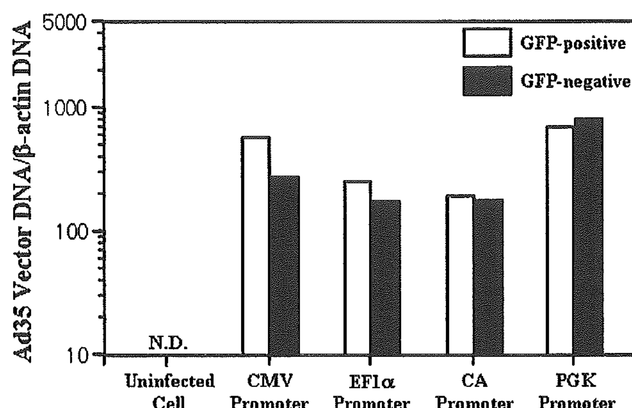

Figure 4 Ad35 vector copy numbers in GFP-positive and -negative cells following Ad35 vector transduction into human bone marrow CD34+ cells. The CD34+ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP-positive and -negative cells were sorted and the total DNA was extracted from the cells. The copy numbers of Ad35 vectors and β-actin were analyzed by Taqman PCR. All data represent the means of two independent experiments. ND, not detected (under the limit of detection).

Transduction in immature subpopulations of human bone marrow CD34+ cells

Next, to examine the promoter activities in the primitive hematopoietic subpopulations among the CD34+ cells, $CD34^+CD38^{low/-}$ cells and $CD34^+AC133^+$ cells were transduced with the Ad35 vectors. $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ cells are known to be the more primitive subsets among the CD34+ cells.[25–27] Transduction experiments demonstrated that the CA, EF1α, and CMVi promoters were clearly superior in $CD34^+CD38^{low/-}$ subsets compared with the CMV, MSCV, and PGK promoters (Figure 5). Similar results were obtained for $CD34^+AC133^+$ subsets (Figure 6). Among these three promoters, use of the CA promoters resulted in the highest transgene expression in both $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ subsets (57% GFP-positive for $CD34^+CD38^{low/-}$ subsets, 51% GFP-positive for $CD34^+AC133^+$ subsets). These data indicate that the CA, EF1α, and CMVi promoters mediate the higher transduction efficiencies and that the CA promoter is the most efficient in these immature CD34+ subpopulations.

CA promoter activity in the colony-forming hematopoietic progenitors

To evaluate the gene expression potential of the CA promoter in colony-forming hematopoietic progenitors, the transduced cells were sorted into GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter, and colony-forming assays were then performed for the sorted GFP-positive and -negative cells. Values indicating the colony-forming unit (CFU) content of the sorted cells 14 days after plating are shown in Table 1. Comparing the total number of colonies derived from the GFP-positive cells with those from the uninfected cells and the GFP-negative cells, it appears that the GFP-positive cells formed almost the same total numbers of colonies as the uninfected cells and the GFP-negative cells. CFU-granulocyte-macrophage (CFU-GM) colonies were grown without significant reduction from the CA promoter-mediated GFP-positive cells, compared with

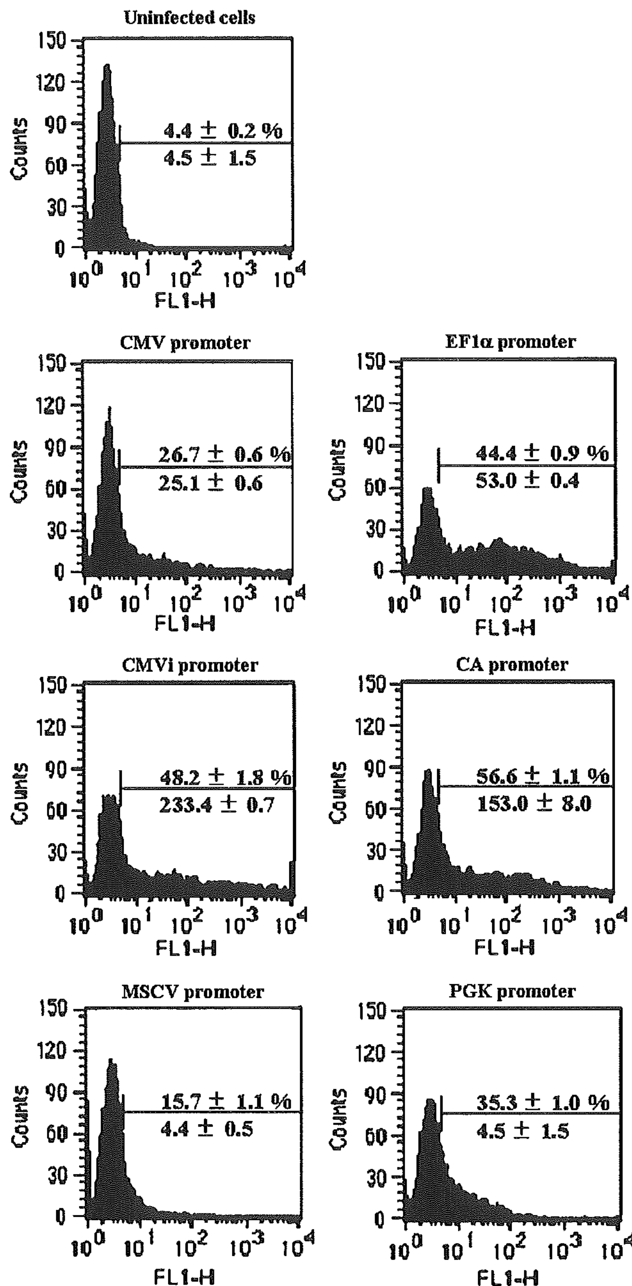

Figure 5 Comparison of promoter activities in the $CD34^+CD38^{low/-}$ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The $CD34^+CD38^{low/-}$ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of two experiments.

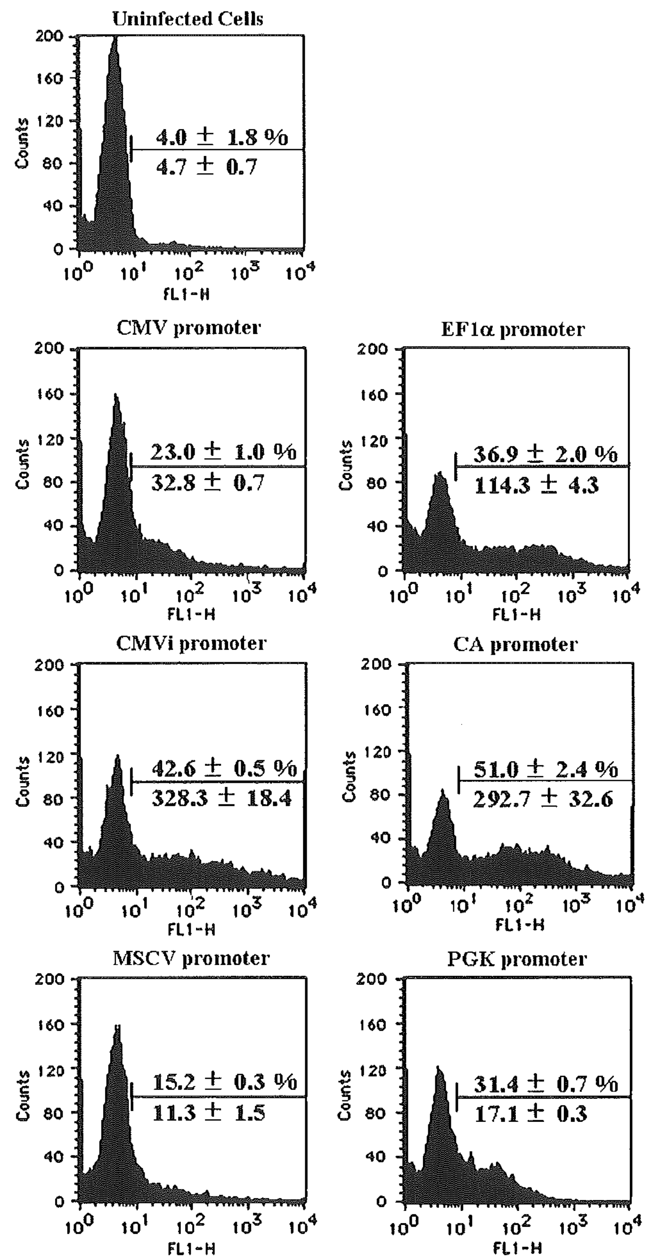

Figure 6 Comparison of promoter activities in human bone marrow $CD34^+AC133^+$ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The $CD34^+AC133^+$ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of two experiments.

the GFP-negative cells, although the growth of burst-forming units-erythroid (BFU-E) colonies was slightly impaired in the GFP-positive cells. CFU-granulocyte erythrocyte monocyte macrophage (CFU-Mix) colonies, which are derived from the most primitive hematopoietic progenitors, were also found in GFP-positive cells. These data suggest that the CA promoter would be significantly active in immature colony-forming progenitors. However, the size of colonies from both GFP-positive and -negative cells appeared to be smaller than that in uninfected cells (data not shown), suggesting that exposure to Ad35 vectors slightly impairs the growth of colony-forming bone marrow hematopoietic progenitors under these conditions.

Discussion

The choice of a promoter to drive transgene expression is important in gene transfer experiments. Currently, a few systematic examinations of promoter activities have been carried out in gene transfer experiments *in vivo*[28–30] and

Table 1 Numbers of colonies derived from GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter in human CD34+ cells

CD34+ cells	*Total*	*BFU-E*	*CFU-GM*	*CFU-Mix*
Sample 1				
Uninfected cells	222.8±25.5	72±11.8	150.3±14.2	0.5±0.6
GFP-positive	193.5±29 (86.9%)	61±9.9 (84.7%)	131.5±17.7 (87.5%)	1±1.4
GFP-negative	180.5±13.4 (81%)	24.5±0.7 (34%)	155.5±13.4 (103.5%)	0.5±0.7
Sample 2				
Uninfected cells	124.8±13.5	44.5±6.1	78.8±9.5	1.5±0.6
GFP-positive	115±11.3 (92%)	29±5.7 (65.2%)	85.5±6.4 (108.5%)	0.5±0.7
GFP-negative	158±19.8 (127%)	26±0 (58.4%)	130.5±20.5 (165.6%)	1.5±0.7

The data represent the mean number of colonies±s.d. from duplicate cultures and the percentage of number of colonies/uninfected cells.

in vitro;[19,29,31] however, information regarding the promoter activities in HSCs is limited and controversial. The aim of this study was to identify an Ad35 vector platform for efficient transgene expression in human HSCs by optimizing a promoter that directs transgene expression. For this purpose, we constructed a series of Ad35 vectors in which GFP expression was driven by a variety of promoters and compared the levels of GFP expression in human bone marrow CD34+ cells and immature CD34+ subsets.

In the present study, we examined the following promoter activities in human bone marrow CD34+ cells: the CMV, EF1α, CMVi, CA, MSCV, and PGK promoters, which are widely used in transduction experiments. Comparison of the six types of promoters showed that a significant increase in GFP-positive cells was obtained with the EF1α, CMVi, and CA promoters. Among these three promoters, the CA promoter was the most efficient at transducing human bone marrow CD34+ cells (Figure 3) and immature CD34+ subsets (CD34+CD38low/− and CD34+AC133+ subsets) (Figures 5 and 6) under the condition employed. Furthermore, the CA promoter-mediated GFP-positive cells formed nearly the same numbers of colonies as uninfected cells in the colony assay (Table 1). The powerful activity of the CA promoter has been demonstrated in important target cells for gene therapy, including dendritic cells,[32] lymphocytes,[32] and hepatocytes.[33] In addition, earlier work with the CA promoter noted heightened transgene expression in immature cells. Okabe *et al*[34] have demonstrated that the CA promoter functions in the embryos of transgenic mice. Efficient transgene expression has also been achieved with the CA promoter in murine embryonic stem cells.[35,36] The data described above indicate that the CA promoter would be the promoter of choice for high levels of transgene expression in immature cells, including HSCs/progenitors.

The CMV promoter is one of the strongest promoters and is currently in wide use for transient gene expression experiments. However, the CMV promoter did not mediate high levels of GFP expression in the CD34+ cells (Figure 2b), immature subsets (Figures 5 and 6), and colony-forming CD34+ progenitors (data not shown). Several groups have demonstrated that the CMV promoter does not allow for high transduction efficiencies in human CD34+ cells[3,19] and murine embryonic stem cells,[36,37] suggesting that the CMV promoter might not be appropriate for immature cells. However, the inclusion of intron A into the CMV promoter (CMVi promoter) largely increases GFP expression in the CD34+ cells as well as immature CD34+ subsets. The β-actin intron is also included in the CA promoter. These data suggest that an intron may be a key element for efficient transgene expression in human CD34+ cells, although a detailed mechanism for enhancement of transgene expression by intron A in CD34+ cells has not yet been elucidated.

Recently, the human membrane cofactor protein CD46 has been shown to be a cellular receptor for subgroup B Ad, including Ad35.[11,12] CD46 is a single-chain type I transmembrane glycoprotein that is expressed in almost all human cells.[13,14] The ubiquitous expression of CD46 leads to a broad tropism of Ad35 vectors toward human cultured cells compared with Ad5 vectors.[23] Expression levels of CD46 on the cells appear to correlate with the affinity of Ad35 for the cells, which is similar to the relationship between CAR expression levels on the cells and the transduction efficiencies of Ad5 vectors, as reported below. Segerman *et al*[9] have demonstrated almost 100% binding of Ad35 to human hematopoietic cell lines, Jurkat, K562, and HL-60 cells, which express sufficient levels of CD46.[38,39] The transduction efficiencies of the chimeric Ad5F35 vector, which is an Ad5-based vector containing an Ad35 fiber shaft and knob, have been found to increase progressively with CD46 expression density on a panel of CHO cells stably expressing CD46.[40] Shayakhmetov *et al*[5] have reported the efficient attachment and internalization of Ad35 in human bone marrow CD34+ cells, which express high level of CD46 (Figure 2a). In this study, however, the transduction efficiencies in the CD34+ cells unexpectedly did not increase proportionally with the increased dose of Ad35 vectors, despite the high expression levels of CD46 (Figure 2b). Even the CA promoter, which was the most efficient in this study, did not mediate more than 70% of GFP-positive cells at the increased dose (data not shown). The limitation of the transduction efficiencies in the CD34+ cells is likely due to the promoter activities that direct transgene expression. We have demonstrated that similar amounts of Ad35 vector genome could be detected in both GFP-positive and -negative cells (Figure 4). These results suggest that Ad35 vectors would interact with all of the CD34+ cells via CD46; however, the promoters examined here do not function in all infected CD34+ cells. It is well known that human CD34+ cells are heterogeneous. With the GFP-negative cells

that were infected by the Ad35 vectors, there may not be sufficient enough levels of the transcriptional factors for promoter function. Transduction with Ad35 vector containing a more suitable promoter might result in much higher transduction efficiencies in human $CD34^+$ cells. A similar phenomenon was observed in the study by Shayakhmetov *et al*,[5] in which human $CD34^+$ cells were transduced with the chimeric Ad5F35 vector. The genome of the chimeric Ad5 vector was detected in both GFP-positive and -negative cells.

Segerman *et al*[41] demonstrated that there are two different receptors for Ad35 in human cells. One is CD46 and another receptor is currently unidentified. It remains to be clarified whether human $CD34^+$ cells express the unidentified receptor for Ad35 and whether the unknown receptor plays an important role on Ad35 vector-mediated transduction in the $CD34^+$ cells.

A number of studies have evaluated the transduction efficiencies in $CD34^+CD38^{low/-}$ cells by staining the transduced $CD34^+$ cells with anti-CD38 antibody just before flow-cytometric analysis, not before transduction.[6,42,43] However, in this study, sorted $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ subpopulations were transduced with Ad35 vectors to evaluate the transduction efficiencies in these immature $CD34^+$ subsets because the expression levels of CD38 in the $CD34^+$ cells would decrease during culture, irrespective of the mature/immature state. Dorrell *et al*[44] and Donaldson *et al*[45] have reported that a dramatic increase in $CD34^+CD38^-$ cell frequency occurred during culture; however, these cells lost the potential to repopulate in the NOD/SCID mouse and to form colonies in the colony-forming assay, respectively. We confirmed that the expression levels of CD38 and AC133 decreased after a 2-day culture (data not shown). As such, the $CD34^+$ cells might have to be sorted before transduction to truly evaluate the transduction efficiencies in primitive hematopoietic subsets. In addition, because the $CD34^+$ cells may differentiate during culture and Ad35 vectors may infect differentiated cells efficiently, Ad35 vectors in the present study were incubated with the $CD34^+$ cells for 6 h and the cells were then washed to remove Ad35 vectors. More efficient transduction with Ad35 vectors was achieved when the $CD34^+$ cells were continuously cultured with Ad35 vectors for 48 h (data not shown), as reported previously.[10]

Various methods have been established for feasible generation of Ad vectors.[46] Among these methods, the improved *in vitro* ligation method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method based on plasmid construction. To efficiently construct a series of Ad35 vectors, an improved *in vitro* ligation system was applied to construction of replication-incompetent E1/E3-deleted Ad35 vectors. We have previously constructed Ad35 vector plasmids by gel purification of DNA fragments and two-step *in vitro* ligation.[10] Reddy *et al*[47] have also constructed Ad35 vector plasmids by several steps of *in vitro* ligation of DNA fragments. Vogels *et al*[48] and Gao *et al*[49] have reported construction of recombinant Ad35 vectors by homologous recombination in 293-derived cell lines and PER.C6 cells stably expressing Ad35 E1B 55K, respectively. All of these methods described above are time-consuming and inefficient. Using the improved *in vitro* ligation method, we more rapidly obtained yields of Ad35 vectors similar to those reported previously.[10] In addition, based on the sequence information of the Ad35 E1 and E3 regions, the E1 deletion size has been increased and most of the E3 region has been deleted in pAdMS4, leading to an increase in the packaging capacity. The increase in the deletion size in the E1 and E3 regions did not reduce the transduction efficiencies of Ad35 vectors (data not shown). Further deletions in the E1 and E3 coding regions must make it possible to insert larger foreign genes into the Ad35 vectors.

As the Ad genome does not integrate into the host genome, transgene expression via Ad vectors can occurr transiently, which is suitable for *ex vivo* manipulation of HSCs and the study of gene functions. This property also results in a low risk of insertion mutagenesis, but stable transgene expression is not allowed. To address this limitation, several groups have developed hybrid Ad vectors such as Ad/adeno-associated virus vectors, which can integrate viral genome into the host genome.[50–52] The Ad35 vector would be a promising framework for the development of these improved vectors.

In summary, we have demonstrated that the EF1α, CMV promoter containing intron A, and the hybrid CA promoter is superior at transducing human bone marrow $CD34^+$ cells in the context of Ad35 vector. In particular, the CA promoter functions most efficiently in $CD34^+$ cells and immature $CD34^+$ subsets. The results of our study provide valuable information regarding gene transfer into HSCs.

Materials and methods

Plasmids

Vector plasmids pAdMS2, -3, and -4 were constructed as follows. The *Sbf*I/*Pst*I fragment of pFS2-Ad35-7,[10] which has the left end of the Ad35 genome (bp 1–367 and 2917–3670) with an E1 deletion, was ligated with the *Sbf*I site of pFS2,[10] resulting in pFS2-Ad35-9. (The end of the *Sbf*I site is compatible with a *Pst*I site.) pFS2-Ad35-9 was cut by *Pac*I and *Bam*HI, and ligated with oligonucleotides 1 (5′-TATAACTATAACGGTCCTAAGGTAGCGA*ATTTAAAT***ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA**-3′) and 2 (5′-GATC**TGCCATTTCATTACCTCTTTCTCCGCACCCGACATAGAT***ATTTAAAT*TCGCTACCTTAGGACCGTTATAGTTATAAT-3′) (I-*Ceu*I, *Swa*I, and PI-*Sce*I recognition sequences are noted by underlining, italics, and bold, respectively), resulting in pFS2-Ad35-10, which contains I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion site of the Ad35 genome. The *Sbf*I/*Pst*I fragments of pFS2-Ad35-7 and pFS2-Ad35-10 were then ligated, resulting in pFS2-Ad35-11. The *Sbf*I/*Asc*I fragment of pFS2-Ad35-11 was exchanged with the *Sbf*I/*Asc*I fragment of pAdMS1,[10] which clones the whole Ad35 genome, resulting in pAdMS2-1. The *Not*I site of pAdMS2-1 was changed into an *Sbf*I site by using oligonucleotide 3 (5′-GGCCCCTGCAGG-3′) (the *Sbf*I recognition sequence is underlined), resulting in pAdMS2. To delete the E3 region in the Ad35 genome, the *Bam*HI/*Not*I fragment of pHM15-Ad35-1,[10] which has the right end of the Ad35 genome (bp 29732 – right end of the genome), was cloned into *Bam*HI/*Not*I sites of a shuttle plasmid pFS1, which contains multicloning sites composed of *Sal*I/*Sbf*I/*Eco*RI/*Bam*HI/*Swa*I/*Not*I sites, creating pFS1-Ad35-1. pFS1 was constructed by ligation of *Xba*I/*Sac*I-digested pGEM7Zf(+) (Promega

Corp., MA, USA) with the oligonucleotides containing the multi-cloning sites. The *Sal*I/*Eco*RI fragment of pHM15-A35-1 (bp 23583-27760) was inserted between the *Sal*I/*Eco*RI sites of pFS1-Ad35-1, resulting in pFS1-Ad35-2. pFS1-Ad35-2 was then cut by *Eco*RI/*Bam*HI and ligated with oligonuclotide 4 (5′-AATTGGCCACG TAGGCC-3′) and 5 (5′-GATCGGCCTACGTGGCC-3′) (*Sfi*I recognition sequence is underlined), resulting in pFS1-Ad35-9. The *Sal*I/*Not*I fragment of pFS1-Ad35-9 was ligated with the *Sal*I/*Not*I fragment of pHM14-Ad35-1, creating pHM14-Ad35-3. pHM14-Ad35-1 was constructed by cloning of the *Eco*RI/*Kpn*I fragment (bp 21945–29545) of the Ad35 genome into the *Eco*RI/*Kpn*I sites of pHM14.[53] The *Eco*RI/*Not*I fragment of pHM14-Ad35-3 was ligated with the *Eco*RI/*Not*I fragment of pAdMS1, resulting in pAdMS3-1. The *Not*I site of pAdMS3-1 was changed into an *Sbf*I site by using oligonucleotide 3, resulting in pAdMS3. The *Sbf*I/*Asc*I fragment of pFS2-Ad35-11 was ligated with the *Sbf*I/*Asc*I fragment of pAdMS3-1, creating pAdMS4-1. The *Not*I site of pAdMS4-1 was changed into a *Sbf*I site by using oligonucleotide 3, resulting in pAdMS4. pAdMS2 and -4 have I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region (ΔE1: bp 368–3374). pAdMS3 and -4 have an *Sfi*I site in the E3 deletion region (ΔE3: bp 27 761–29 731). The E1a and E1b coding regions of Ad35 are located from bp 569 to 1441 and from bp 1611 to 3400, respectively, according to the Ad35 genome sequence (GenBank Accession No. AY271307). The E3a and E3b coding regions of Ad35 are located from bp 27 199 to 29 496 and from bp 29 538 to 30 622, respectively. pAdMS2, -3, and -4 have *Sbf*I sites at both ends of the Ad genome.

Shuttle plasmids containing a variety of promoters were constructed by changing the CMV promoter of pHMCMV5[21] into another type of promoter, including the EF1α promoter, the CA promoter, the mouse PGK promoter, the MSCV promoter, and the CMVi promoter. The EF1α promoter is derived from pEF1α/myc/nuc (Invitrogen, Carlsbad, CA, USA). The CMVi promoter is derived from pGeneGrip (Gene Therapy Systems, San Diego, CA, USA). The composite CA promoter and PGK promoter were kindly provided by Dr J Miyazaki (Osaka University, Osaka, Japan) and Dr MA Kay (Standford University, CA, USA), respectively. The MSCV promoter was a kind gift of Dr RG Hawley (American Red Cross, MD, USA).

E1/E3-deleted Ad35 vectors expressing enhanced GFP

To construct the plasmid for a recombinant E1/E3-deleted Ad35 vector containing a CMV promoter-driven GFP expression cassette, pHMCMV-GFP1[54] and pAdMS4 were digested with I-*Ceu*I and PI-*Sce*I. The digested pAdMS4 was ligated with the I-*Ceu*I/PI-*Sce*I fragment of pHMCMV-GFP1 containing a GFP expression cassette, resulting in pAdMS4-CMVGFP. pAdMS4-CMVGFP was linearized by the digestion with *Sbf*I. The linearized DNA was transfected into VK10-9 cells (kindly provided by Dr V Krougliak),[22] which are 293 cells expressing the E4 proteins of Ad5 as well as the E1 proteins. A cytopathic effect (CPE) was observed 10–14 days after transfection, and the virus was then amplified in VK10-9 cells and purified by the conventional method for Ad5 vector preparation. For the preparation of recombinant E1/E3-deleted Ad35 vectors containing various types of promoters, a *GFP* gene was cloned into multi-cloning sites in the shuttle plasmids containing various types of promoters, and the Ad35 vectors were then prepared by methods similar to those described above.

Transduction experiment

Human bone marrow CD34$^+$ cells were purchased from Biowhittaker, Inc., Walkersville, MD, USA. The cells were recovered from the frozen stock, suspended in StemSpan™ 2000 containing cytokine cocktail StemSpan™ CC100 (human Flt-3 ligand (100 ng/ml), human stem cell factor (100 ng/ml), human interleukin (IL)-3 (20 ng/ml), and human IL-6 (20 ng/ml)) (StemCell Technologies Inc., Vancouver, BC, Canada), and were seeded into a 48- or 96-well plate ($1–5 \times 10^4$ cells/well). The cells were transduced with the GFP-expressing Ad35 vectors at the indicated VP/cell 16–18 h after seeding. At 6 h after incubation, the cells were washed to remove the Ad35 vectors and resuspended in the medium. At 48 h after transduction, 10^4 cells per sample were analyzed for GFP expression by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan).

Flow-cytometric analysis of CD46 expression

Human bone marrow CD34$^+$ cells were suspended in staining buffer containing fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD46 antibody (Pharmingen, San Diego, CA, USA). After washing with the sorting solution, the stained cells (10^4 cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson). For simultaneous analysis of GFP and CD46 expression, the transduced cells were incubated with mouse anti-human CD46 antibody (Pharmingen). Subsequently, the cells were washed and incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG second antibody (Pharmingen). After washing with the sorting solution, the analysis was performed as described above.

Real-time quantitative PCR

Human bone marrow CD34$^+$ cells were incubated with the Ad35 vectors at 6000 VP/cell, and control cells were incubated without the Ad35 vectors. After a 6-h incubation, the medium was changed to remove the Ad35 vectors. At 48 h after transduction, the cells were harvested, pelleted, and washed gently. The cells were then sorted into GFP-positive and -negative fractions using a FACSVantage SE (Becton Dickinson). Sort purities were greater than 90% for both GFP-positive and -negative fractions. The sorted cells were treated with trypsin and DNase, followed by washing to remove the extracellular vector genome. Total DNA, including the Ad35 vector DNA, was extracted from the GFP-positive and -negative cells using a Tissue DNeasy Kit (Qiagen, Valencia, CA, USA). The quantitative real-time PCR was performed with 2.5 ng of sample DNA, 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 μl using the ABI Prism 7000 sequence detection system (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and

60°C for 1 min. The reaction was carried out for 50 cycles. Primers for amplification were located in the pIX region of Ad35 genome. The sequences of the primers and probe used were as follows: forward, 5′-TGGATGGAAG ACCCGTTCAA-3′; reverse, 5′-CGTCCAAAGGTGAAG AACTTAAAGT-3′; probe, 5′-FAM-CGCCAATTCTTC AACGCTGACCTATGC-TAMRA-3′. These sequences were designed using Primer Express softeware version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired size. The Ad35 vector plasmid pAdMS4 was used as a standard. For human β-actin quantification, β-actin control reagent (Applied Biosystems) was used.

Purification of immature CD34$^+$ subpopulations

Human bone marrow CD34$^+$ cells were incubated with PE-conjugated mouse anti-human AC133 monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) or FITC-conjugated mouse anti-human CD38 monoclonal antibody (eBioscience, San Diego, CA, USA) immediately after the cells were recovered from the frozen stock. After washing, cell sorting was performed using a FACSVantage SE (Becton Dickinson). Sorting gates were set to sort the CD38$^{low/-}$ and AC133$^+$ subpopulations. Sort purities were greater than 80% for both CD38$^{low/-}$ and AC133$^+$ subpopulations. The sorted CD34$^+$CD38$^{low/-}$ and CD34$^+$AC133$^+$ cells were transduced with the Ad35 vectors at 6000 VP/cell, as described above.

Colony-forming assay

The GFP-positive and -negative cells were recovered 48 h after transduction with the Ad35 vector containing the CA promoter, as described above. In all, 1000 cells of each fraction were then plated in a 35-mm dish containing Methocult H4444 methylcellulose medium (erythropoietin; 3 U/ml, stem cell factor; 50 ng/ml, GM-CSF; 10 ng/ml, IL-3; 10 ng/ml) (Stem Cell Technologies). After 14 days of incubation at 37°C in a 5% CO_2 incubator, CFU-GM, BFU-E, and CFU-Mix colonies were enumerated under a microscope. The experiments were performed in duplicate. Uninfected cells were also sorted into a GFP-negative fraction and treated as described above.

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