Highly efficient transient gene expression and gene targeting in primate embryonic stem cells with helper-dependent adenoviral vectors

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Human embryonic stem (hES) cells are regarded as a potentially unlimited source of cellular materials for regenerative medicine. For biological studies and clinical applications using primate ES cells, the development of a general strategy to obtain efficient gene delivery and genetic manipulation, especially gene targeting via homologous recombination (HR), would be of paramount importance. However, unlike mouse ES (mES) cells, efficient strategies for transient gene delivery and HR in hES cells have not been established. Here, we report that helper-dependent adenoviral vectors (HDAdVs) were able to transfer genes in hES and cynomolgus monkey (*Macaca fasicularis*) ES (cES) cells efficiently. Without losing the undifferentiated state of the ES cells, transient gene transfer efficiency was ≈100%. Using HDAdVs with homology arms, approximately one out of 10 chromosomal integrations of the vector was via HR, whereas the rate was only ≈1% with other gene delivery methods. Furthermore, in combination with negative selection, ≈45% of chromosomal integrations of the vector were targeted integrations, indicating that HDAdVs would be a powerful tool for genetic manipulation in hES cells and potentially in other types of human stem cells, such as induced pluripotent stem (iPS) cells.

gene delivery | human embryonic stem cell

Embryonic stem (ES) cells have the property of self-renewal and pluripotency to differentiate into various cell types derived from the three embryonic germ layers: the ectoderm, mesoderm, and endoderm (1, 2). Thus, ES cells represent an excellent model to study basic developmental biology, the potential for drug discovery, and an unlimited source of various cell types and tissues for transplantation therapy of many diseases. Mouse ES (mES) cells are the best characterized, in which the methods for genetic modification, including gene targeting via homologous recombination (HR), have been well established, and many murine models of human disease became available. However, human ES (hES) cells differ from mES cells in many aspects such as the expression of cell surface markers and response to leukemia inhibitory factor (LIF) (3–7). Furthermore, ethical, cultural, and legal perspectives are potential barriers against a usage of human ES cells. For these reasons, nonhuman primate ES cells, such as those that we previously established from cynomolgus monkey (*Macaca fasicularis*) (8), provide research tools by which to understand properties and behaviors of hES cells. Because of the phylogenetic closeness between monkeys and humans, cynomolgus monkey ES (cES) and hES cells show similarities, such as cell surface marker expression and low cloning efficiency, making cES cells a useful resource for biological and preclinical research before the clinical usage of hES cells.

However, mainly because of fragility to experimental manipulation of primate ES cells, methodologies to achieve transient gene expression in ≈100% of cells have not been established (9, 10). Furthermore, unlike mES cells, in which gene targeting via HR has been used routinely with great success, there are few studies using gene targeting in hES cells (11–17). Gene targeting study in nonhuman primate ES cells has not yet been reported. Although three investigative groups have reported that *HPRT1* gene targeting was achieved in hES cells by using electroporation (11, 12, 17), the frequencies of HR were extremely low at $\approx 1 \times 10^{-6}$ per cell, and the percentages of targeted to random chromosomal integration were also low (≈2%). Obviously, more efficient gene targeting methods would be required to generate models for therapeutic applications in transplantation medicine and human diseases by using hES cells.

Adenoviral vectors (AdVs) efficiently transduce a broad range of cell types and have been used extensively in preclinical and clinical studies of gene therapy (18, 19). Although an E1-deleted AdV was used for gene transfer into hES cells, the transduction efficiency (11%) was low (20). Helper-dependent AdVs (HDAdVs) were originally developed to overcome host immune responses against E1-deleted AdVs *in vivo* (21, 22). Because of the complete removal of viral genes from the vector genome, HDAdVs are generally less cytotoxic than E1-deleted AdVs, which allows them to be used at higher multiplicities of infection (MOIs) (22). In addition, we previously showed that the expanded cloning capacity of HDAdVs, which permits the insertion of larger segments of homologous DNA for HR, is advantageous in that it obtains highly efficient gene repair via HR in mES cells (23). The frequency of HR were extremely high at $\approx 2.2 \times 10^{-3}$ per cell, and the percentage of HR to random integration was >50%.

In this report, we investigated whether HDAdVs are superior for transferring genes into primate ES cells, and transient gene transfer efficiencies of ≈98% were achieved while maintaining the pluripotency in both cES and hES cells. When HDAdVs with sequences homologous to the host *HPRT1* locus were used, one in 10 chromosomal integrations of the vector was via HR. Furthermore, in combination with negative selection, approximately half of the drug resistant colonies were targeted at the *HPRT1* gene via HR. These results suggest that gene transfer

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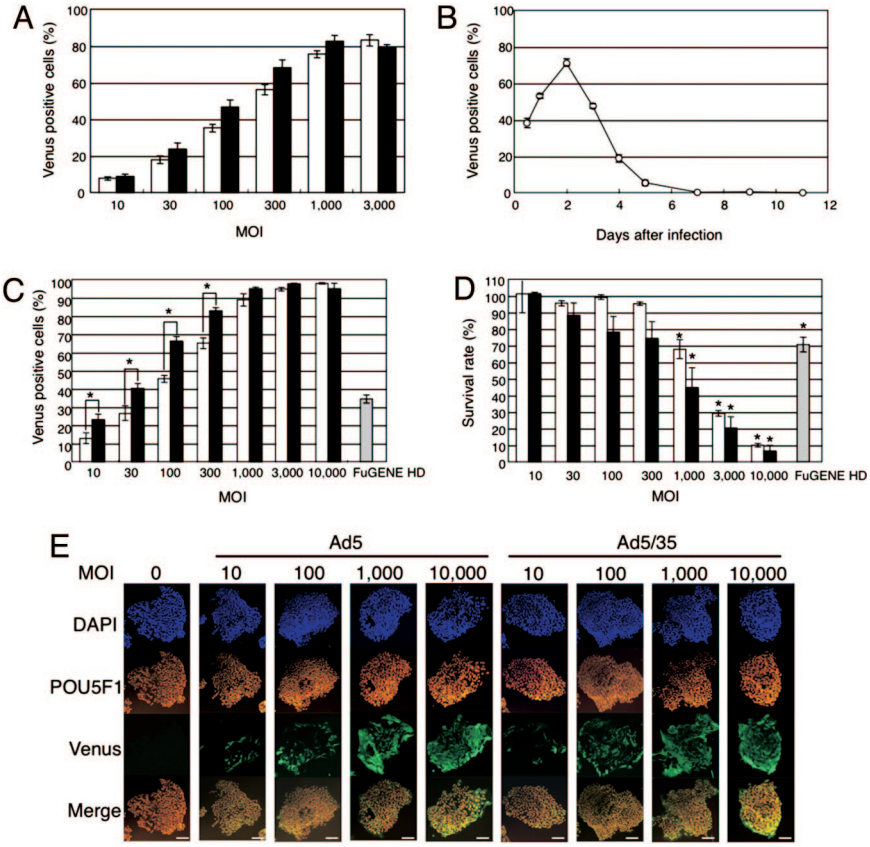

Fig. 1. Transient gene expression in primate ES cells using HDAdVs. (*A*) Transient gene expression efficiencies in cES cells. CMK6 cells were infected with HDAdVenus-geo-TK, pseudotyped with Ad5 (open bars) or Ad5/35 fiber (filled bars), at various MOIs. The average of three independent experiments is shown with a standard error bar. (*B*) Time course of transient gene expression in cES cells. CMK6 cells were infected with HDAdVenus-geo-TK, pseudotyped with Ad5 fiber, at an MOI of 300. The average of three independent experiments is shown with a standard error bar. (*C*) Transient gene expression efficiencies in hES cells. KhES-1 subline 1 cells were infected with HDAdVenus-geo-TK, pseudotyped with Ad5 fiber (open bars) or Ad5/35 fiber (filled bars), at various MOIs or were transduced with pHDAdVenus-geo-TK plasmid DNA using FuGENE HD (gray bar). The average of three independent experiments is shown with a standard error bar. *, $P < 0.05$, *t* test, between Ad5 and Ad5/35 fibers. (*D*) Cytotoxicity after HDAdV infection of hES cells. KhES-1 cells were infected with HDAdVenus-geo-TK, pseudotyped with Ad5 (open bars) or Ad5/35 fiber (filled bars), at various MOIs or were transduced with pHDAdVenus-geo-TK plasmid DNA using FuGENE HD (gray bar). The average of three independent experiments is shown with a standard error bar. *, $P < 0.05$, *t* test, between noninfected and infected cells. (*E*) Expression of stem cell markers in HDAdV-infected hES cells. KhES-1 cells were infected with HDAdVenus-geo-TK, pseudotyped with Ad5 or Ad5/35 fiber, at various MOIs. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). The merge shows costaining of Venus and POU5F1. (Scale bars, 200 μm.)

mediated by HD AdVs would be a powerful technology for genetic manipulation in primate ES cells.

Results

Transient Gene Expression in cES Cells. To examine the applicability of HDAdVs for gene expression in cES and hES cells, we first investigated the efficiency of transient gene expression with HDAdVs. We constructed pHDAdVenus-geo-TK, an HDAdV DNA containing the Venus gene, which encodes a brighter mutant of the yellow fluorescent protein (24). This HDAdV DNA was packaged into virus particles with human adenovirus type 5 (Ad5) fiber or Ad5/35 fiber, the knob and shaft domains of which are derived form the Ad35 fiber (25). The former utilizes the coxsackievirus B-adenovirus receptor (CAR) as a primary cellular attachment receptor (26), whereas the later uses CD46 (27). cES cells (CMK6, XY male karyotype) (8) were infected at various MOIs, which were determined as Venus-transducing units measured on 293 cells, ranging from 10 to 3,000 cells. Venus-positive cells were detected by FACS 2 days after infection. Transient gene expression efficiency was ≈10% at an MOI of 10 and was as high as 83% at MOIs of 1,000 to 3,000 (Fig. 1*A*). There was no significant difference (*t* test, $P > 0.05$) in efficiencies between the HDAdVs with the Ad5 fiber and the Ad5/35 fiber. To examine the time course of transient gene expression with HDAdVs in cES cells, Venus-positive cells were analyzed by FACS at time points ranging from 12 h to 11 days after infection. Gene expression peaked at 2 days after infection and was hardly detectable 7 days after infection (Fig. 1*B*), indicating strong but transient gene expression in dividing ES cells as expected. To assess whether HDAdV-infected cells maintained ES cell characteristics, we examined colocalization of the undifferentiated ES cell marker Pou5f1 (Oct3/4) and the Venus proteins by immunostaining in cES cells, which were infected at MOIs of 30–3,000 (data not shown). Pou5f1 expression was observed in almost all Venus-positive cells, suggesting that the undifferentiated state was maintained after HDAdV infection.

Transient Gene Expression in hES Cells. We then investigated if hES cells (KhES-1 subline 1, XX female karyotype) (28) can also be efficiently transduced with HDAdV. In addition, we measured survival rates of hES cells after HDAdV-mediated gene transfer. The results showed an MOI-dependent increase in transient gene expression efficiency (Fig. 1*C*) and cytotoxicity (Fig. 1*D*). For infection at low MOIs (10–300), the efficiency of HDAdV with Ad5/35 fiber was significantly (*t* test, $P < 0.05$) higher than

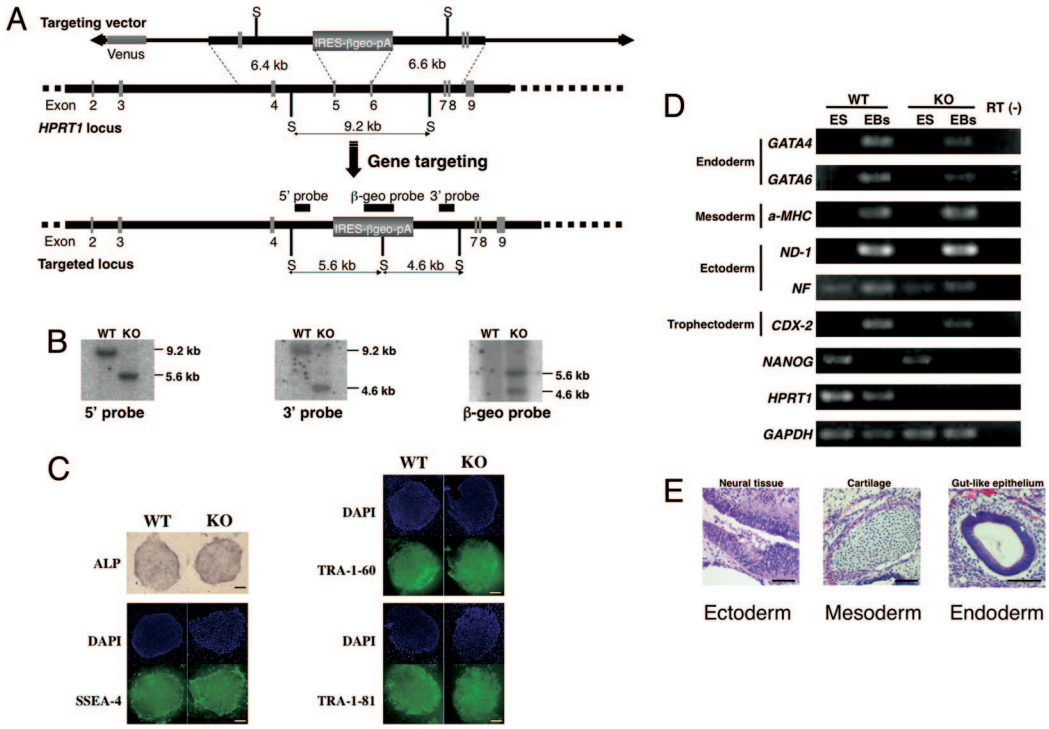

Fig. 2. Gene targeting of the *HPRT1* locus in cES cells. WT, noninfected wild-type CMK6 cells; KO, *HPRT1*-knockout cES cells. (*A*) Structures of the HDAdV targeting vector, the cynomolgus monkey *HPRT1* locus, and the targeted locus. The probes for Southern blot analyses are shown as black bars. Venus, the Venus expression cassette driven by CS2 promoter; IRES, internal ribosomal entry site; βgeo, a fusion of the β-galactocidase and the neomycin resistant genes; pA, SV40 polyadenylation signal; S, *Sac*I sites. (*B*) Analysis of the *Sac*I-digested genomic structure at the cynomolgus monkey *HPRT1* locus by Southern hybridization. (*C*) Expression of stem cell markers in the *HPRT1*-knockout cES cells. ALP, alkaline phosphatase. (Scale bars: 200 μm.) (*D*) Multipotency of the *HPRT1*-knockout cES clone. The cells were induced to form EBs *in vitro* and analyzed by RT-PCR for expression of the following lineage-specific markers: *GATA4* and *GATA6* for endoderm; α-myosin heavy chain (*α-MHC*) for mesoderm; neurogenic differentiation-1 (*ND-1*) and neurofilament 68 kDa (*NF*) for ectoderm; *CDX2* for trophectoderm; *NANOG* for undifferentiated cES cells. *HPRT1* mRNA expression disappeared in *HPRT1*-knockout cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. ES, embryonic stem; EBs, embryoid bodies; RT (−), PCR analysis without reverse transcriptase. (*E*) *In vivo* differentiation of a *HPRT1*-knockout cES clone. Hematoxylin and eosin staining of teratoma formed after injection of *HPRT1*-knockout cES cells into a SCID mouse. Tissues derived from three germ layers were found. (Scale bars, 100 μm.)

that of HDAdV with Ad5 fiber, and compared with noninfected cells, no significant cytotoxicity (*t* test, $P > 0.05$) was observed. At an MOI of 1,000, the efficiency was ≈90% with some decrease in cell number at the same level as that of FuGENE HD-transfected cells. At higher MOIs (3,000–10,000), the HDAdVs were able to infect 95–98% of hES cells. Although the infected cells maintained the undifferentiated state, as detected by anti-POU5F1 immunostaining (Fig. 1*E*), the survival rates of the infected hES cells were significantly reduced (*t* test, $P < 0.05$). The gene expression efficiencies of HDAdVs were higher than that of the nonviral transfection reagent FuGENE HD (34%), which was the most efficient with low cytotoxicity among >30 transfection reagents tested (29). These results suggest that HDAdVs would be a powerful tool for transient gene expression in cES and hES cells.

Gene Targeting of the *HPRT1* Locus in cES Cells. We reported previously that, by encoding long homologous sequences, HDAdV is an efficient and versatile gene targeting vector in mouse ES cells (23). To examine the efficiency of gene targeting by using HDAdVs in primate ES cells, we constructed a cynomolgus monkey *HPRT1*-targeting HDAdV plasmid, pBRHDAd-rHPRTKO5-R, encoding a 13-kb region homologous to introns 3–8 of *HPRT1*, in which exons 5 and 6 were replaced with the IRES-βgeo-pA cassette (Fig. 2*A*). The internal ribosomal entry site (IRES) permits the direct cap-independent translation of the β-geo reporter gene, which encodes a fusion protein with neomycin phosphotransferase (*neo*) and β-galactosidase (β-gal) activities. This construct would increase the ratio of targeted to random integration, as the splice accepter signal at the 5′ end of this cassette allows the expression of the promoterless *neo* gene only when it integrates within transcriptionally active loci (30). This HDAdV DNA was packaged into virus particles with the Ad5 fiber. Because the *HPRT1* gene is located on the X chromosome, a single gene replacement via HR leads to complete loss of the HPRT1 activity in male XY cells and makes the cells 6-thioguanine (6TG) resistant. When 8.0×10^5 ES cells were infected with the HDAd-rHPRTKO5-R vector under an optimized condition with an MOI of 30, three G418-resistant colonies were obtained, one of which was G418/6TG double-resistant. The fidelity of gene targeting for this clone was confirmed by Southern analysis (Fig. 2*B*). The *HPRT1*-knockout ES cells expressed the alkaline phosphatase and ES surface markers (SSEA-4, TRA-1–60, TRA-1–81) at the same levels as the noninfected cES cells, suggesting the undifferentiated state was not affected by HDAdV-mediated gene targeting (Fig. 2*C*). We also investigated the differentiation potential of the *HPRT1*-knockout ES cells into all three germ layers *in vitro* and *in vivo*. The expression of marker genes specific for undifferentiated and differentiated states was analyzed by reverse transcription-PCR (RT-PCR) in embryoid bodies (EBs) derived from the *HPRT1*-knockout cES cells (Fig. 2*D*). Although expression of the undifferentiated state specific marker (*NANOG*) was reduced in EBs, the differentiation marker genes for endoderm (*GATA4* and *GATA6*), mesoderm (α-myosin heavy chain), ectoderm (neurogenic differentiation-1 and neurofilament 68 kDa), and

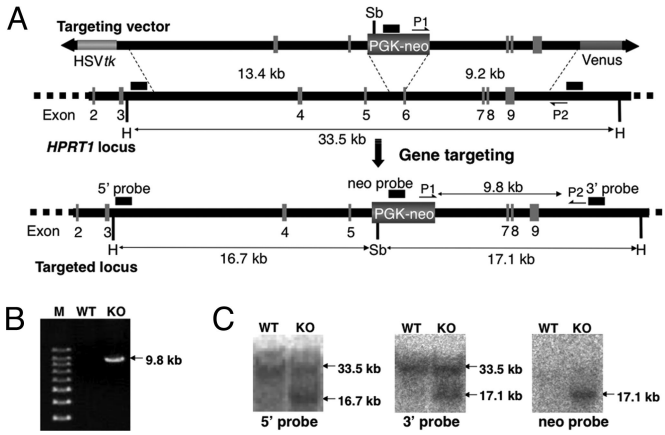

Fig. 3. Gene targeting of the *HPRT1* locus in hES cells. WT, noninfected wild-type KhES-1 cells; KO, *HPRT1*-knockout hES cells. (*A*) Structures of the HDAdV targeting vector, the human *HPRT1* locus, and the targeted locus. The probes for Southern blot analyses are shown as black bars. Arrows (P1 and P2) indicate a pair of primers for PCR analysis. HSV*tk*, the herpes simplex virus thymidine kinase gene expression cassette driven by MC1 promoter; PGK-neo, the neomycin-resistant gene expression cassette driven by PGK promoter; Venus, the Venus expression cassette driven by CS2 promoter; H, *Hpa*I sites; Sb, *Sbf* I sites. PCR analysis (*B*) and Southern blot analysis (*C*) at the *HPRT1* locus. Because KhES-1 is a female cell line with two X chromosomes, HR resulted in one band corresponding to the targeted allele and another from the residual unmodified allele. M, Kb DNA Ladder (STRATAGENE).

trophectoderm (*CDX2*) were increased in *HPRT1*-knockout cES cells in the same pattern as that of the noninfected cES cells. To analyze differentiation potentials *in vivo*, we transplanted noninfected and *HPRT1*-knockout cES cells into severe combined immunodeficiency (SCID) mice. Teratomas, analyzed 3 months after transplantation, contained all three germ layers (Fig. 2*E*). These results indicate that cES cells maintain pluripotency after gene targeting with HDAdVs.

Gene Targeting of the *HPRT1* Locus in hES Cells. Next, to examine the efficiency of gene targeting in hES cells, we constructed a human *HPRT1*-targeting HDAdV plasmid (pBRHDAd-hHPRT-PGKneoF), containing longer homology arms of 14.3 kb and 9.2 kb on each side (Fig. 3*A*). The gene-trap strategy, which was used to improve the targeted to random integration ratio for *HPRT1* knockout in cES cells, is not applicable for gene targeting at transcriptionally inactive loci. Therefore, to evaluate general usefulness of HDAdVs for gene targeting in primate ES cells, the plasmid encoded the *neo* gene cassette driven by the PGK promoter. To enrich the gene targeting ratio, the plasmid also contained the MC1 promoter-driven HSV*tk* (herpes simplex virus thymidine kinase) gene, which allows negative selection with ganciclovir (GANC). This *HPRT1* targeting DNA was packaged into virus particles with Ad5 fiber. As a control, we electroporated the linearized pBRHDAd-hHPRT-PGKneoF plasmid into hES cells (KhES-1) under optimal conditions (11). After a several sets of electroporation of a total of 1.1×10^8 cells, 172 G418-resistant clones (1.6×10^{-6}/cell) were obtained. Among them, only one clone was targeted to the *HPRT1* locus, indicating the gene targeting frequency of 9.1×10^{-9} (the percentage of targeted to random integration was 0.58%), suggesting that electroporation is inefficient. A total of 5.1×10^6 cells were infected with the HDAdV at an MOI of 300, and 136 G418-resistant colonies were obtained (2.7×10^{-5}/cell). Among them, 31 colonies were GANC-resistant, and thus the frequency of G418/GANC double-resistant clones was 6.1×10^{-6}/cell. PCR (Fig. 3*B*) and Southern blot analysis (Fig. 3*C*) demonstrated that 14 of these 31 colonies (45%) had been precisely targeted at the *HPRT1* gene via HR, indicating the gene targeting frequency of 2.7×10^{-6}, which is higher than that by electroporation by ≈300-fold. There was no ectopic vector integration in all of the targeted clones (data not shown). We also confirmed that *HPRT1*-targeted hES cells maintained ES cell characteristics, such as an undifferentiated state [supporting information (SI) Fig. S1] and pluripotency (data not shown).

Subsequently, we examined gene targeting efficiency in another human ES line, KhES-3 (XY male karyotype) (31), which shows remarkably more fragility to experimental manipulation and lower replating efficiency than the KhES-1 (28). A total of 1.2×10^6 ES cells were infected with the vector at an MOI of 300, and seven G418-resistant colonies were obtained (5.8×10^{-6}/cell). Among them, three colonies were GANC-resistant. 6TG-resistance and PCR analysis demonstrated that one of these three colonies had been targeted at the *HPRT1* gene via HR (data not shown). These results indicate that HDAdV can be used for gene targeting in a variety of primate ES cell lines. Furthermore, HDAdV-mediated gene targeting requires much fewer cells than electroporation.

Discussion

The highest gene transfer efficiency in hES cells is reported to be as great as 85% using nucleofection (10). The efficiency with HDAdVs in hES cells was >90% at an MOI of 1,000 with cytotoxicities similar to that by FuGENE HD. At higher MOIs, it was 95–100%, which is the highest among all previous reports, including both nonviral and viral methods, with some decrease in cell number but without losing the undifferentiated state. These results suggest that HDAdVs are especially suitable for experiments in which gene transfer in nearly 100% of hES cells is required, such as gene knock-down using shRNA and inducible gene expression systems.

Although the chromosomal integration frequencies of HDAdVs in primate ES cells were low (≈2.7×10^{-5}/cell), the ratio of targeted to random integration using HDAdVs was higher compared with that of nonviral electroporation, which is consistent with our previous observation in mES cells (23). Although the ratio was 1:10 with HDAdV-mediated gene targeting even without negative selection, it was 1:50–600 for *HPRT1* gene targeting by electroporation (11, 12, 17). Furthermore, in combination with negative selection, approximately half of the drug-resistant colonies (14 of 31) were targeted at the *HPRT1* gene via HR. The hES cells are technically much harder to maintain in culture and to keep undifferentiated than are mouse ES cells, because of characteristics such as slow growth, a low plating efficiency, and insensitivity to LIF (6, 7). Because it is demanding to screen many hES clones, especially in the case of gene targeting experiments, it would be advantageous to use HDAdVs for gene targeting, which shows a high ratio of targeted to random integration. Efficient HR with HDAdVs can be potentially combined with subsequent efficient transient expression of Cre recombinase mediated by HDAdVs to remove loxP sites present in the gene targeting sequences, resulting in highly successful gene knock-in applications in hES cells. Finally, by taking advantages of viral vector-mediated gene transfer, gene-targeted clones could be obtained from fewer cells ($1\sim5 \times 10^6$) even without cell dissociation.

Two major recombination pathways, HR and nonhomologous end-joining (NHEJ), have been identified as repairing cellular double-stranded breaks, which are the most detrimental DNA lesions. It is believed that gene targeting and random integration is mediated by HR and NHEJ, respectively. Therefore, the reason for the high ratio of relative gene targeting with HDAdVs is either activation of HR and/or repression of NHEJ by HDAdV-mediated gene transfer. The termini of adenoviral genomes incoming into cell nuclei are protected by the terminal protein (TP), which is believed to prevent chromosomal inte-

gration of adenoviral genomes. NHEJ factors, such as the Ku70-Ku80 complex that binds to free ends of double-stranded DNA, might only weakly interact with the ends of the HDAdV genome; and, as a result, NHEJ-mediated random integration might be relatively rare. It is also possible that the association of the HDAdV genome with TP or unknown proteins bound to the vector genome may recruit HR proteins. Further analyses will be required to elucidate the mechanism of the high relative targeting rate.

An adeno-associated virus vector is also a promising vector for gene targeting and has been applied to correct dominant mutations in mesenchymal stem cells from patients of osteogenesis imperfecta (32, 33). Compared with adeno-associated virus vectors, HDAdVs are more efficient in gene delivery into hES cells (20), show a higher ratio of targeted to random integration, and accommodate larger and more complex targeting cassettes.

Recently, extremely high levels of gene targeting (5.3%) without selection in hES cells were reported by using a combination of zinc-finger nucleases (ZFNs), which can cleave chromosomes at any target site, and integrase-defective lentiviral vectors (14). To achieve ZFN-mediated gene targeting, codelivery of two genes encoding ZFNs and donor DNA is a major hurdle. HDAdVs might also be suitable for a ZFNs-mediated gene targeting strategy in hES cells, because of highly efficient transient gene expression in nearly 100% of hES cells, highly efficient gene targeting, and the large cloning capacity of HDAdVs, which allows the incorporation of two ZFNs and donor DNA into one vector.

Several groups recently reported that iPS cells can be generated from human fibroblasts (34–37). Human iPS cells may replace hES in some medical applications, because iPS technology allows creation of patient-specific stem cells from adult humans. HDAdVs may also be applicable for genetic modification of human iPS cells, inasmuch as various characteristics are similar between these cell types.

Materials and Methods

Construction of HDAdVs. To construct the Venus-expressing HDAdV, the CAG promoter-driven Venus gene was subcloned into the HDAdV plasmid, pHDAd-geo-TK (our unpublished data), to make pHDAdVenus-geo-TK. To generate the cynomolgus monkey *HPRT1* targeting vector, we isolated isogenic homologous DNA containing the cynomolgus monkey *HPRT1* gene by long-distance genomic PCR. The targeting vector was designed by replacing exons 5–6 of the *HPRT1* gene with the IRES-βgeo-pA cassette, and then subcloned into the HDAdV plasmid, pBRHDAd-TV (our unpublished data), to make pBRHDAd-rHPRTKO5-R. To generate the human *HPRT1* targeting vector, we first isolated a BAC clone containing the human *HPRT1* gene (CalTech Human BAC Clone, BHS1214). By using the RED/ET recombination technique (38), 1.2-kb of DNA, including a part of intron 5 and exon 6 of the *HPRT1* gene, was replaced with the PGK-EM7-neobpA cassette, and then subcloned into the HDAdV plasmid, pBRHDAd-TK-Venus (our unpublished data), to make pBRHDAd-hHPRT-PGKneoF. A detailed description of these subclonings will be provided on request.

Preparation of HDAdVs. After transfection of *Pme* I-linearized HDAdV plasmids into 293Cre66 cells (kindly provided by Drs. Kochanek and Schiedner) in the presence of helper virus, AdNG163 (39), the rescued vectors were propagated by serial passages on 116 cells with the addition of AdNG163 helper virus (Ad5 fiber) or AdHPBGF35 helper virus (Ad5/35 fiber) (40) at each passage, as described in ref. 39. HDAdVs were purified with two rounds of CsCl density gradient ultracentrifugation, as described in ref. 41. Throughout this study, infectious vector titers were defined as the Venus-transducing units measured on 293 cells, since the infection efficiencies of 293 cells between the vectors with the Ad5 fiber and the Ad5/35 fiber were nearly equal (42).

Measurement of Transient Gene Expression Efficiencies. The hES cell lines (KhES-1 subline 1 and KhES-3) were used following the hES cell research guidelines of the Japanese government. Cultures of the cES cell line (CMK6) and the hES cell lines were maintained as described (8, 31). The ES cells were plated onto six-well dishes coated with Matrigel (BD) in mouse embryonic fibroblast (MEF)-conditioned medium on day 1. On day 2, the cells were infected or transfected as follows. For HDAdV infection, the culture medium was replaced by 200 μl of Dulbecco's modified Eagle's medium (DMEM) F-12 (Sigma), the cells were counted and infected with the vectors at various MOIs for 1 h at room temperature, and 2 ml of MEF-conditioned medium was added. For plasmid DNA transfection, the culture medium was replaced by fresh MEF-conditioned medium, and the cells were transfected with 2 μg of pHDAdVenus-geo-TK plasmid using FuGENE HD (Roche) according to the manufacturer's instructions. To measure transient gene expression efficiency on day 4, the cells were dissociated, resuspended in ES culture medium including 1 μg/ml propidium iodide (PI), and analyzed on the FACS Calibur flow cytometer (BD). The samples were gated on a forward scatter-side scatter gate to exclude cell debris and subsequently on a PI gate to exclude the necrotic PI-positive cells. To measure cytotoxicity on day 4, viable cell numbers were counted by PI exclusion by using NucleoCounter (ChemoMetec A/S), and the survival rates were calculated by dividing the number of viable infected cells by the number of viable noninfected cells. These experiments were performed more than three times and statistical significances were determined using the *t* test.

Measurement of *HPRT1*-Knockout Efficiencies. For infection with *HRPT1* targeting HDAdVs, the ES cells were plated onto 100-mm dishes coated with Matrigel on day 1, and cultured with MEF-conditioned medium. On day 2, the culture medium was replaced by 1 ml of DMEM F-12, the cells were counted and infected with the *HRPT1* targeting vectors at various MOIs for 1 h at room temperature, and 8 ml of MEF-conditioned medium was added. For plasmid DNA electroporation, we followed the optimal conditions as reported (11). G418 selection (50 μg/ml; Nacalai tesque) was started 2 days after infection or electroporation. After 10 days, the G418 concentration was doubled to 100 μg/ml. After 3 weeks, surviving colonies were transferred to 96-well plates and GANC selection (2 μM; Invitrogen) was started. 2.5 μM and 10 μM 6TG (Sigma) selections were also started in CMK6-derived and KhES-3-derived cells, each of which have an XY male karyotype. These 6TG-resistant clones were analyzed by Southern blot analyses. For KhES-1-derived cells, G418 and GANC double-resistant clones were characterized by both PCR and Southern blot analyses.

Immunocytochemistry and Immunocytofluorescence Analysis. For assessing the undifferentiated state of *HPRT1*-knockout ES cells, the expression of alkaline phosphatase and surface markers (SSEA-4, TRA-1–60, and TRA-1–81) was examined by immunocytochemical staining with the ES cell characterization kit (CHEMICON) following the manufacturer's protocol. For Venus and Pou5f1 immunofluorescence staining, the infected cells were cultured for 2 days without a feeder layer on glass slides and fixed with 4% paraformaldehyde for 20 min. After permeabilization with 0.2% Triton X-100 for 10 min, the cells were incubated with anti-Oct-3/4 mouse monoclonal antibody (clone C-10, 1:300; Santa Cruz) and anti-GFP rabbit antibody (1:300; Molecular Probes) in PBS containing 2% bovine serum albumin and 3% goat serum at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG (IgG) antibody (Molecular Probes) and Alexa Fluor 546-conjugated anti-mouse IgG antibody (Molecular Probes) at room temperature for 1 h. Cells were washed three times with PBS, mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories), and examined under a fluorescence microscope.

Formation of EBs and RT-PCR. The ES cells were detached from the feeder cells by enzymatic dissociation with gentle pipetting to avoid the dissociation of colonies. The ES cells were then cultured in suspension in a 100-mm Petri dish. EBs were grown in DMEM (Nacalai tesque) supplemented with 15% (vol/vol) Knockout Serum Replacement (Invitrogen) for 20 days, and then collected for preparation of total RNA. Total RNA was extracted from ES cells using the NucleoSpin RNAII kit (Macherey-Nagel), according to the manufacturer's protocol. cDNA was synthesized from 4 μg of total RNA using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa). The PCRs were optimized to allow semiquantitative comparisons within the log phase of amplification. *NANOG* and *HPRT1* gene-specific primers were designed based on published sequences as follows: *NANOG* (570 bp), #542: 5′-TACCTCAGCCTCCAGCAGATGCAAGAAC-3′; and #541: 5′-GTCGACTCACACATCTTCAGGTTGCATG-3′; *HPRT1* (516 bp), #640: 5′-ATGCTGAGGATTTGGAAAGGGTGTTTATTC-3′; and #641 5′-TGAAGTATTCATTATAGTCAAGGGCATATC-3′. The sequences of other gene-specific primers were described (43, 44).

Teratoma Formation. $\approx 10^7$ ES cells were injected s.c. into SCID mice (CLEA Japan). After 2 to 3 months, the resulting teratomas were dissected, fixed, and stained with hematoxylin and eosin, as described in ref. 31. Animal protocols were approved by the Institutional Board on Animal Care.

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