

Efficient and Accurate Homologous Recombination in hESCs and hiPSCs Using Helper-dependent Adenoviral Vectors

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Low efficiencies of gene targeting via homologous recombination (HR) have limited basic research and applications using human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Here, we show highly and equally efficient gene knockout and knock-in at both transcriptionally active (*HPRT1*, *KU80*, *LIG1*, *LIG3*) and inactive (*HB9*) loci in these cells using high-capacity helper-dependent adenoviral vectors (HDAdVs). Without the necessity of introducing artificial DNA double-strand breaks, 7–81% of drug-resistant colonies were gene-targeted by accurate HR, which were not accompanied with additional ectopic integrations. Even at the motor neuron-specific *HB9* locus, the enhanced green fluorescent protein (EGFP) gene was accurately knocked in in 23–57% of drug-resistant colonies. In these clones, induced differentiation into the *HB9*-positive motor neuron correlated with EGFP expression. Furthermore, HDAdV infection had no detectable adverse effects on the undifferentiated state and pluripotency of hESCs and hiPSCs. These results suggest that HDAdV is one of the best methods for efficient and accurate gene targeting in hESCs and hiPSCs and might be especially useful for therapeutic applications.

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INTRODUCTION

Increasing the efficiency of gene targeting in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) is essential to improve both the experimental and therapeutic potential of these cells. Although gene targeting by electroporation has been routinely used in mouse embryonic stem cells (mESCs), its application in hESCs/hiPSCs has been limited.^{1,2} The ratio of targeted to random chromosomal integration (the “relative” targeting efficiency) in these cell types by electroporation is generally low (0–2%).^{3–7} Recently, high relative targeting

efficiencies were reported in hESCs/hiPSCs by electroporating modified bacterial artificial chromosomes (BACs), encoding long homologous DNA to the target sequences.⁸ Zinc-finger nucleases (ZFNs) are also used for highly efficient gene targeting at both transcriptionally active and inactive loci.^{9–11} However, the accuracy of homologous recombination (HR) by these methods, which is critical for the therapeutic application of iPSCs, has not yet been proven. Viral vectors have been utilized to overcome the low DNA delivery and gene-targeting efficiencies in hESCs/hiPSCs.^{9,12,13} A high-capacity helper-dependent adenoviral vector (HDAdV)^{14,15} recently yielded relative targeting efficiencies of ~45% at the hypoxanthine phosphoribosyltransferase (*HPRT1*) locus in hESC lines, thus suggesting its potential as a universal gene-targeting vector in human pluripotent stem cells.¹⁶

The current study expanded the general applicability of HDAdVs to gene knockout and knock-in at several loci, both transcriptionally active and inactive, in hESCs as well as in hiPSCs. Efficiencies of accurate gene targeting were 7–81% at five loci without detectable effects on the undifferentiated state and pluripotency. Importantly, 75–100% of homologous recombinants were produced by accurate HR without additional vector integration at ectopic sites, suggesting high fidelity and the potential safety of HDAdV-mediated gene targeting.

RESULTS

Gene knockout of *HPRT1* locus in hiPSCs

Gene targeting at the *HPRT1* locus in hESCs was efficiently achieved using HDAdV (Table 1).¹⁶ The current study first targeted the *HPRT1* locus in the male hiPSC lines, 246H1 and 246G1, using the same *HPRT1*-targeting HDAdV (Figure 1a).¹⁷ HR, which knocks out the sole allele on the X chromosome, is detected by selecting the infected cells with 6-thioguanine-resistance (6TG^R). The cells were also subjected to negative selection with ganciclovir (GANC) to improve the relative gene-targeting efficiency because the vector also encodes the *HSVtk* gene. HDAdV infection of 246G1 cells resulted in 135 G418-resistant (G418^R) colonies, 79 of which were G418^R-GANC-resistant (GANC^R). Of those G418^R-GANC^R

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Table 1 Summary of gene-targeting efficiencies in human ESCs and iPSCs

Cell line	Locus	Delivery method	Treated cells	G418 ^R colonies	G418 ^R -GANC ^R colonies	Targeted integration ^a	Accurate gene targeting			
							Confirmed/analyzed ^b	Per cell ^c	Per G418 Rd	Per G418 Rd GANC Rd
KhES-1 ^e		HDAdV	5.1 × 10 ⁶	136	31	14 (10%)	14/14 (100%)	2.7 × 10 ⁻⁶	10%	45%
KhES-3 ^e		HDAdV	1.9 × 10 ⁶	14	6	2 (14%)	2/2 (100%)	1.1 × 10 ⁻⁶	14%	33%
KhES-1 ^e	<i>HPRT1</i>	EP	1.1 × 10 ⁸	172	98	1 (1%)	N.D.	9.1 × 10 ⁻⁹	0.6%	1%
246G1		HDAdV	2.5 × 10 ⁷	135	79	16 (12%)	5/5 (100%)	6.4 × 10 ⁻⁷	12%	20%
246H1		HDAdV	5.1 × 10 ⁶	39	14	1 (3%)	1/1 (100%)	2.0 × 10 ⁻⁷	3%	7%
246H1		EP	2.1 × 10 ⁸	126	93	0 (0%)		0		
KhES-3	<i>HB9</i>	HDAdV	5.0 × 10 ⁶	172	21	12 (7%)	12/12 (100%)	2.4 × 10 ⁻⁶	7%	57%
246H1		HDAdV	1.7 × 10 ⁶	280	61	14 (5%)	3/3 (100%)	8.2 × 10 ⁻⁶	5%	23%
246H1	<i>LIG3</i>	HDAdV	4.4 × 10 ⁶	164	73	41 (25%)	3/4 (75%)	7.0 × 10 ⁻⁶	19%	42%
246H1	<i>LIG1</i>	HDAdV	4.4 × 10 ⁶	93	28	12 (13%)	4/5 (80%)	2.2 × 10 ⁻⁶	10%	34%
246H1	<i>KU80</i>	HDAdV	2.2 × 10 ⁶	227	153	124 (55%)	3/3 (100%)	5.6 × 10 ⁻⁵	55%	81%
Summary of HDAdV-mediated gene targeting										
4	5	9 experiments		1,260	466	3–55%	47/49 (75–100%)	2.0 × 10 ⁻⁷ to 5.6 × 10 ⁻⁵	3–55%	7–81%

EP, electroporation; ESCs, embryonic stem cells; GANC, ganciclovir; HDAdV, helper-dependent adenoviral vector; HPRT1, hypoxanthine phosphoribosyltransferase; iPSCs, induced pluripotent stem cells; N.D., not determined.

^aG418^R-GANC^R colonies with the vector integrated at the target locus was determined by PCR analyses. ^bAccuracy of gene targeting was determined by Southern analyses. ^cThe absolute (per cell) targeting efficiencies were calculated as (the number of targeted integration) × (% confirmed by Southern analyses) / (the number of treated cells). ^dThe relative (per chromosomal integration) targeting efficiencies were calculated as (the number of targeted integration) × (% confirmed by Southern analyses) / (the number of drug-resistant colonies). ^eThese data were previously published.¹⁶

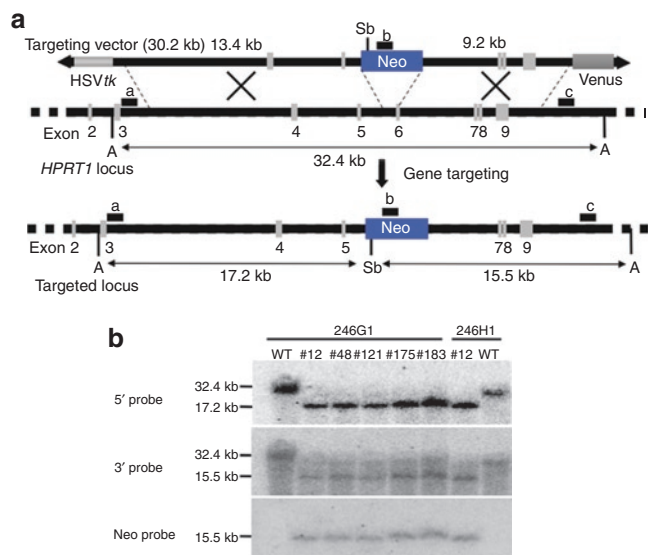


Figure 1 Gene targeting at *HPRT1*. (a) Schematic illustration of *HPRT1* knockout with HDAdV. The probes for Southern analyses are shown as black bars. a, 5' probe; b, neo probe; c, 3' probe. HSVtk, the herpes simplex virus thymidine kinase gene cassette; Neo, the neomycin-resistant gene cassette; Venus, an expression cassette for the Venus (F46L mutant yellow fluorescent protein gene ref. 41); A, *AhdI* sites; Sb, *SbfI* sites. (b) Southern analyses of wild type (WT) and the *HPRT1*-knockout human induced pluripotent stem clones. Genomic DNA was digested with *AhdI* and *SbfI*. HDAdV, helper-dependent adenoviral vector.

colonies, 16 were also 6TG^R. The resultant gene-targeting efficiency (G418^R-GANC^R-6TG^R colonies)/(G418^R-GANC^R colonies) was 20% with positive-negative selection (Table 1). A similar efficiency of 7% was observed in the 246H1 cell line (Table 1). The linearized

HPRT1-targeting HDAdV plasmid was electroporated into the 246H1 cells as a control, resulting in no HR out of 126 G418^R clones. Southern hybridization (Figure 1b) demonstrated that those 6TG^R clones had been targeted accurately at the *HPRT1* locus via HR, without ectopic vector integration (Supplementary Figure S1a). These results indicate that HDAdV-mediated gene targeting is equally efficient in both hESCs and hiPSCs.

Heterozygous knockout of *KU80*, *LIG1*, and *LIG3* genes in hiPSCs

Next, to examine the applicability of HDAdV-mediated gene targeting at other housekeeping loci, the Lupus Ku autoantigen protein p80 (*KU80*) locus of the nonhomologous end-joining (NHEJ) pathway, and the DNA Ligase I (*LIG1*) and DNA Ligase III (*LIG3*) loci of the potential backup pathway of NHEJ were targeted. NHEJ is a major pathway involved in DNA double-strand break (DSB) repair. *KU70* and *KU80* are the key factors in NHEJ, and the heterozygous mutant cells or RNAi-induced knockdown of Ku resulted in elevated gene-targeting frequencies via HR in the HCT116 and NALM-6 human somatic cell lines.^{18,19} The 246H1 cells were infected with gene-targeting HDAdVs that were designed to delete exons 3–4 of *KU80*, exons 3–4 of *LIG1*, and exons 5–7 of *LIG3* (Figures 2a and 3a,b). Detailed Southern analyses of G418^R and GANC^R clones revealed relative gene-targeting efficiencies of 81% at *KU80*, 34% at *LIG1*, and 42% at *LIG3* (Figures 2b and 3c, Supplementary Figure S1b–d, Table 1). Importantly, in contrast to ZFN-mediated gene targeting, no ectopic vector integration was observed in any of these targeted clones (Supplementary Figure S1b–d). In these heterozygous hiPS clones, the inserted neomycin-resistant gene (*neo*) was flanked by the mutant *loxP* sites (*lox71* and *lox66*). After transfection of pCAGGS-Cre, which encodes the

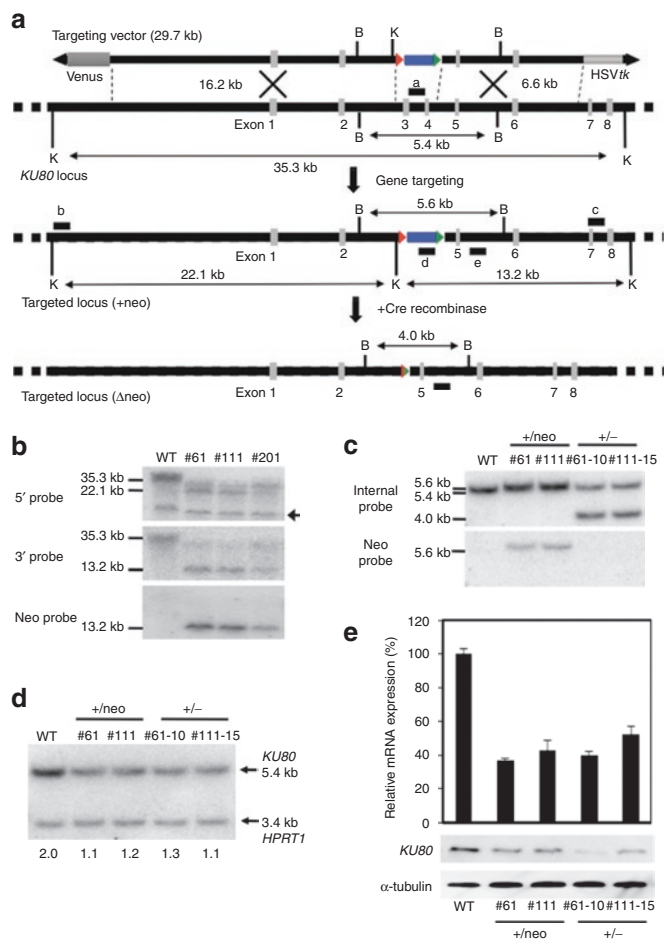


Figure 2 Gene targeting at *KU80*. **(a)** Schematic illustration of *KU80* heterozygous knockout with HDAdV. The probes for Southern analyses are shown as black bars; a, neo probe; b, 5' probe; c, 3' probe; d, deleted-region probe; e, internal probe. HSVtk, the herpes simplex virus thymidine kinase gene cassette; Blue box, the neomycin-resistant gene cassette; Venus, the Venus gene cassette; Triangle, loxP site; B, BglII sites; K, KpnI sites. **(b)** Southern analyses of WT (246H1) and heterozygous knockout clones (#61 and #111). #201 is an inaccurate recombinant. Genomic DNA was digested with KpnI. An arrow indicates a nonspecific band. **(c)** Southern hybridization analyses of the *KU80* heterozygous knockout clones obtained from 246H1, and the clones from which the PGKneo cassette was removed by transient Cre expression. The genomic DNA was digested with BglII and hybridized to the ³²P-labeled internal probe or neo probe. WT, wild-type parental cells. **(d)** Quantitative Southern hybridization. Genomic DNA was digested with BglII and hybridized with probe "d." A DNA fragment from intron 3 of *HPRT1* locus was used as a control probe. The copy number of wild-type *KU80* allele determined by a densitometric analysis is indicated. **(e)** Reduction of *KU80* gene expression in the heterozygous mutants. The upper panel indicates the mRNA level determined by quantitative RT-PCR. The average of three independent experiments is shown with the standard deviation. The lower panel indicates the protein level determined by Western blot. An anti- α -tubulin antibody was used as a loading control. HDAdV, helper-dependent adenoviral vector. RT-PCR, reverse transcription-PCR.

CAG promoter-driven Cre gene, and recloning of the cells, *neo* was removed in ~25% of the cells (Figures 2c and 3d, Supplementary Figure S1b–d, Supplementary Table S1). Two independent clones from *LIG1*, *LIG3*, and *KU80* heterozygous mutant iPSCs were subjected to further analyses. The reduction in the copy number of wild-type (WT) allele and of gene expression levels in these

heterozygous hiPS clones were confirmed by quantitative genomic Southern analyses, quantitative reverse transcription-PCR (RT-PCR), and western blotting (Figures 2d,e and 3e,f). These results therefore indicate that highly efficient gene targeting can be achieved at various housekeeping loci using HDAdVs.

Gene knock-in of *HB9* locus in hESCs and hiPSCs

Next, to examine the efficiency of HDAdV-mediated gene targeting at transcriptionally inactive loci, reporter hESC and hiPSC lines were established, in which the enhanced green fluorescent protein (EGFP) gene was knocked into the *HB9* locus by HR. *HB9* is expressed selectively in motor neurons and also in pancreatic cells, but not in ES cells.^{20–22} A human *HB9*-targeting HDAdV was constructed with homology arms of 12.1 kb and 2.0 kb on each side (Figure 4a). The infected hESC line, KhES-3, had 21 GANC^R colonies out of 172 G418^R (Table 1). Southern hybridization demonstrated that 12 of these 21 colonies (57%) had been accurately targeted at the locus, without ectopic vector integration (Figure 4b, Supplementary Figure S1e,f). In addition to hESCs, the *HB9* locus was also targeted in the hiPSC line, 246H1, at similar efficiencies (Table 1, Figure 4b). A karyotype analysis was performed for the knock-in hESC clones G1 and 47, and no abnormality was observed by G-banding (Supplementary Figure S2). We also performed a similar analysis for the *HPRT1*-knockout hESC clone, which we reported previously, and found no abnormalities (Supplementary Figure S2), *HB9*-targeted hESCs and hiPSCs maintained an undifferentiated state (Figure 5a) and pluripotency (Figure 5b,c), as we have previously reported for the *HPRT1*-knockout hESC clones.¹⁶ These results indicate that HDAdV-mediated gene targeting is equally efficient regardless of the transcriptional activities of target loci.

The *HB9*-EGFP knock-in hiPSC line was induced to differentiate into motor neurons to validate differentiation-specific expression of the knocked-in EGFP gene in these reporter cell lines. The *neo* cassette, which is sandwiched by loxP sites, was excised from the knock-in iPSC clone by transient expression of Cre recombinase (Figure 4a) because the presence of drug-resistant gene may interfere with regulated expression of the reporter gene. After transfection with the pCAGGS-Cre plasmid, followed by analyses of G418 sensitivity and PCR, it was confirmed that the drug-resistant cassette was excised from the *HB9* locus in ~50% of the cells (Supplementary Table S1).

The immunofluorescence analysis of *HB9*-EGFP knock-in hiPSC and hESC lines revealed a correlation between EGFP and *HB9* expression (Figure 4c, Supplementary Figure S3). All of the EGFP-positive cells were *HB9*-positive by immunostaining, suggesting the EGFP gene was precisely knocked into the target chromosomal site by HDAdV-mediated gene targeting. Together, these results demonstrated that HDAdVs are a powerful tool for efficient and accurate gene knockout and knock-in in hESCs and hiPSCs.

DISCUSSION

Various attempts have been made to improve gene-targeting efficiencies in hESCs/hiPSCs. Four methods have been reported,

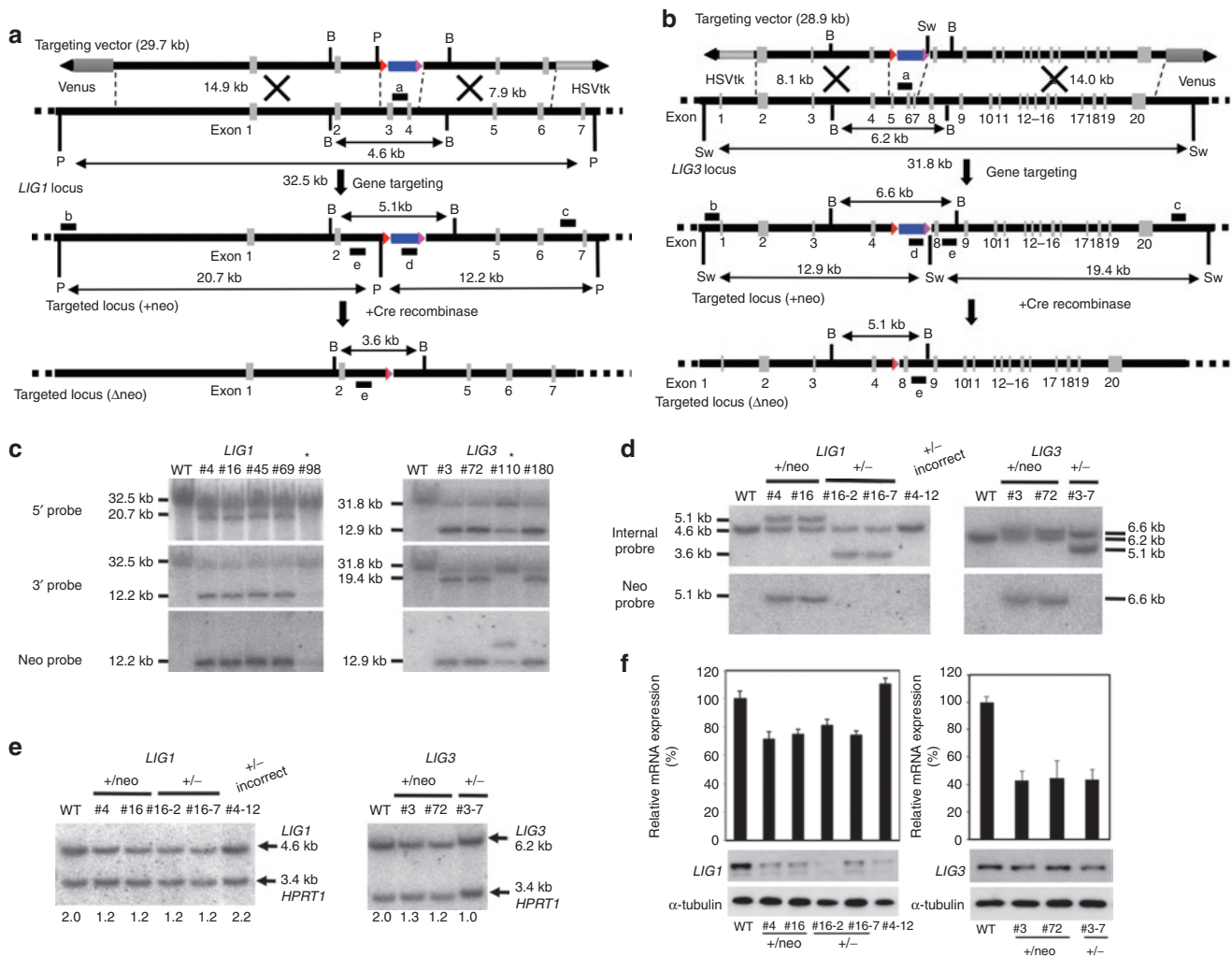


Figure 3 Generation of *LIG1* and *LIG3* heterozygous mutants in human induced pluripotent stem cells. **(a)** Schematic illustration of the *LIG1* heterozygous knockout with HDAdV. The probes for Southern hybridization analyses are shown as black bars; a, neo probe; b, 5' probe; c, 3' probe; d, deleted-region probe; e, internal probe. HSVtk, the herpes simplex virus thymidine kinase gene cassette; Blue box, the neomycin-resistant gene cassette; Venus, the Venus gene cassette; Triangle, loxP site; B, *Bgl*II; P, *Pac*I. **(b)** Schematic illustration of the *LIG3* heterozygous knockout in human induced pluripotent stem cells. The probes for Southern hybridization analyses are shown as black bars; a, neo probe; b, 5' probe; c, 3' probe; d, deleted-region probe. HSVtk, the herpes simplex virus thymidine kinase gene cassette; Neo, the neomycin-resistant gene cassette; Venus, the Venus gene cassette; Red triangle, loxP site; B, *Bgl*II; Sw, *Swa*I. **(c)** Southern hybridization analyses of WT, *LIG1*, and *LIG3* heterozygous knockout clones (*LIG1*: #4, #16, #45, and #69; *LIG3*: #3, #72, and #180). Genomic DNA was digested with *Pac*I or *Swa*I. Asterisks indicate inaccurate homologous recombinants. **(d)** Southern hybridization analyses of the *LIG1* and *LIG3* heterozygous knockout clones obtained from 246H1 cells and clones in which the PGKEM7neo cassette was removed by transient expression of Cre recombinase to confirm the insertion and removal of the PGKneo cassette. The genomic DNA was digested with *Bgl*II and hybridized with the ³²P-labeled internal probe or neo probe. #4-12 is an inaccurate recombinant. WT, wild-type parental cells. **(e)** Quantitative Southern hybridization. Genomic DNA was digested with *Bgl*II and hybridized with probe "d." A DNA fragment from intron 3 of the *HPRT1* locus was used as a control probe to normalize the amount of loaded DNA. The copy number of wild-type *LIG1* or *LIG3* allele determined by a densitometric analysis is indicated. **(f)** Reduction of *LIG1* (left panel) or *LIG3* (right panel) gene expression at mRNA and protein levels in the heterozygous *LIG1* or *LIG3* mutants. The upper panel indicates the mRNA level determined by quantitative RT-PCR. The average of three independent experiments is shown with the standard deviation. The lower panel indicates the protein level determined by Western blotting. An anti- α -tubulin antibody was used as a loading control. HDAdV, helper-dependent adenoviral vector. RT-PCR, reverse transcription-PCR.

which routinely achieve high relative targeting efficiencies in hESCs/hiPSCs. The first method is electroporation of BAC-based gene-targeting constructs with large homologies.⁸ The relative gene-targeting efficiencies were ~33% at three housekeeping loci. However, gene targeting at transcriptionally inactive loci has not yet been reported. The second method utilizes the ZFNs, which are fusion proteins of sequence-specific zinc-finger DNA-binding domains and a nonspecific *Fok*I nuclease domain.^{23,24} Carefully designed ZFNs introduce a chromosomal DSB at the target site,

which greatly enhances the frequency of HR repair near the DSB.²⁵ Several groups have validated the use of ZFNs to target chromosomal loci in hESCs/hiPSCs with high relative targeting efficiencies of 6–100%.^{9–11} However, frequent DSBs at off-target sites, accompanied by inaccurate NHEJ repair and ectopic integration of gene-targeting cassettes, have been observed after ZFN treatment in hESCs/hiPSCs and in other cell types.^{10,11,26–28} The third method utilizes adeno-associated virus (AAV) vectors, with the relative targeting efficiencies of 1–30%.^{12,13,29} The gene-targeting

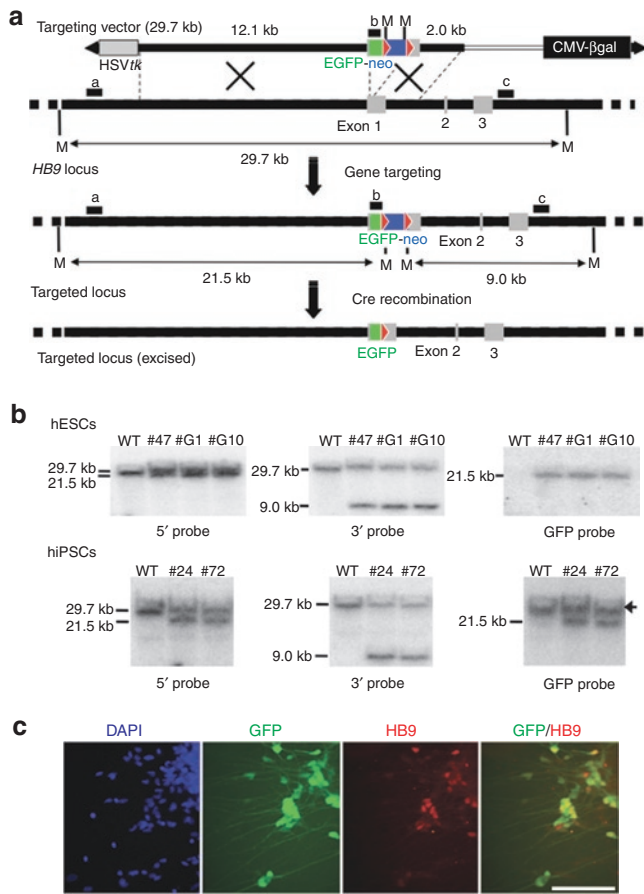


Figure 4 Generation of *HB9-EGFP* knock-in reporter cell lines from hESCs and hiPSCs. **(a)** Schematic illustration of *HB9-EGFP* knock-in with HDAdV. The probes for Southern analyses are shown as black bars. a, 5' probe; b, EGFP probe; c, 3' probe. Green box, the EGFP gene. Blue box, the neomycin-resistant gene. Red triangle, *loxP* site. White bar, stuffer DNA for adjusting the vector size. CMV- β gal, the β gal expression cassette; M, *MfeI* sites. **(b)** Southern analyses of wild-type parental and the *HB9-EGFP* knock-in clones (hESC: #47, #G1, and #G10; hiPSC: #24 and #72). Genomic DNA was digested with *MfeI*. An arrow indicates a signal from the “endogenous” GFP gene, which was encoded by a retroviral vector used for induction of iPSCs. **(c)** Immunofluorescence analysis of motor neurons differentiated from the *HB9-EGFP* knock-in hiPSC clone (#24). Bar, 100 μ m. EGFP, enhanced green fluorescent protein; HDAdV, helper-dependent adenoviral vector; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells.

efficiency can also be evaluated by the efficiency per treated cell (we call this the “absolute” targeting efficiency). The absolute targeting efficiency by the BAC-based method is generally low, at 5×10^{-8} to 5×10^{-7} per cell. In the case of the ZFN-mediated method, the absolute efficiencies were $\sim 7 \times 10^{-5}$ per cell.¹¹ Homozygous mutant hESCs/hiPSCs could also be obtained by a one-step process.¹⁰ The absolute efficiencies by the AAV-based method range from 1.9×10^{-7} to 2.4×10^{-4} per cell.²⁹ While both the relative and absolute targeting efficiencies are high, the AAV vectors have limitations for certain applications, such as the small cloning capacity of ~ 4.7 kb and the relative inefficiency of targeting silent loci.²⁹ In addition to these methods, oligonucleotide-mediated gene modification might be another efficient method, which achieved the absolute efficiencies of $\sim 2 \times 10^{-4}$ per cell in

mouse ESCs,³⁰ although its application to hESCs/hiPSCs has not been reported.

The fourth method involves delivering gene-targeting cassettes using HDAdV. In this study, we demonstrated the versatility of HDAdV in a variety of gene knockouts (*HPRT1*, *LIG1*, *LIG3*, and *KU80*) and knock-ins (*HB9*) in multiple hESC and hiPSC lines. *KU80*, *LIG1*, and *LIG3* heterozygous mutant hiPSCs will be useful for analyzing the biological functions of the genes in human pluripotent stem cells and, potentially, to achieve improved efficiencies of gene targeting in these mutant hiPSCs. A knock-in of a fluorescent marker gene into the hepatocyte-specific *ALB* locus also showed similar targeting efficiencies in hESCs/hiPSCs (manuscript in preparation). Our results and those of a recent report on the *LMNA* locus³¹ indicate that, as in the case of ZFN-mediated HR, HDAdV-mediated HR is not affected by the transcriptional activities of the target chromosomal loci. The efficiencies of accurate gene targeting varied depending on the target locus and the cell line but remained consistently high. In this study, to determine the accuracy of HR, the candidate targeted clones were screened by PCR, followed by Southern analyses. Long homology arms hamper detailed Southern analyses to examine the accuracy of HR by BAC-mediated methods. Additional ectopic integrations have been observed in ZFN-mediated targeted hES and hiPS clones.¹⁰ HDAdVs yielded 3–55% of chromosomal integration of the vectors (G418^R colonies) at the target loci, based on PCR analyses (Table 1). These numbers indicate strong affinity of gene-targeting cassettes delivered by HDAdVs with homologous chromosomal sequences even without artificial DNA DSBs. Detailed Southern analyses with multiple probes and enzymes confirmed that 75–100% of these site-specific integration events were mediated by accurate HR (Supplementary Figure S1). Similar detailed analyses of the structure of integrated gene-targeting cassettes delivered by electroporation into the *HPRT* locus in mouse ES cells reveal that only 5% of 6TG^R colonies are generated by accurate HR,³² indicating high fidelity of HDAdV-mediated gene targeting. Overall, the relative gene-targeting efficiencies were 3–55% and 7–81% of G418^R colonies and G418^R/GANC^R colonies, respectively. Furthermore, gene targeting was equally efficient even at a transcriptionally silent target. Although the cells were maintained under drug (G418 and GANC) selection, deleterious effects were not observed, at least by the analyses of the karyotype, pluripotency, and growth rates of the cells (Figure 5, Supplementary Figure S2, and data not shown). More data from other loci will be needed to elucidate why the gene-targeting efficiency is particularly high at the *KU80* locus. Importantly, in contrast to ZFN-mediated gene targeting, no additional ectopic chromosomal integrations of the vectors were detected in any of the 47 HDAdV-mediated homologous recombinants (Table 1). Inaccurate homologous recombinants (Figure 3c,d) might have been produced by the mechanism described by Adair *et al.*³³ The absolute gene-targeting frequencies with HDAdVs were 2.0×10^{-7} to 5.6×10^{-5} per infected cell (9.0×10^{-6} on average). Therefore, depending on the target locus, the absolute efficiency with HDAdVs is as high as that obtained by using ZFNs (see above). This also indicates that a 100-mm dish of starting hiPSCs ($\sim 3 \times 10^6$ cells) is sufficient to obtain multiple gene-targeted clones. Furthermore, in contrast to

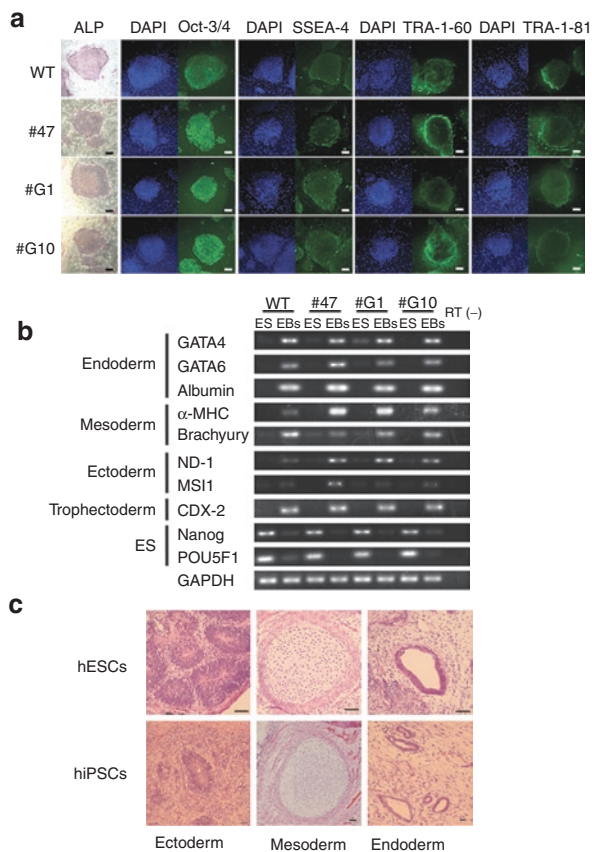


Figure 5 Characterization of *HB9*-EGFP knock-in hESC and hiPSC clones. **(a)** Expression of stem cell markers in the *HB9*-EGFP knock-in hESC clones (#47, #G1, and #G10). ALP, alkaline phosphatase. Bars, 200 μ m. **(b)** Multipotency of the *HB9*-EGFP knock-in hESC clones. Embryoid bodies (EBs) derived from the hESCs were analyzed by RT-PCR for expression of the lineage-specific markers. *GAPDH* was used as a control. RT (-), without reverse transcriptase. **(c)** *In vivo* differentiation of *HB9*-EGFP knock-in hESC (#47) and hiPSC (#24) clones. Tissues derived from three germ layers are indicated. Bars, 50 μ m. EGFP, enhanced green fluorescent protein; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; RT-PCR, reverse transcription.

electroporation, which requires ten million cells for each attempt, HDAdV-mediated gene targeting is equally efficient even with a smaller number of starting cells,³⁴ which minimizes the number of passages/cell divisions to obtain homologous recombinants.

HR has to be accurate for applications such as establishment of gene knock-in iPSC lines to obtain tissue-specific expression of an inserted marker gene upon differentiation. Furthermore, unintended mutations during chromosomal manipulation are obviously not acceptable for therapeutic applications of hESCs/hiPSCs. The characteristics of highly efficient gene-targeting strategies indicate that BAC-mediated gene targeting might be useful for making heterozygote mutants at housekeeping loci because the procedure is relatively simple. ZFN-mediated gene targeting would be useful for making marker gene knock-ins or homozygous mutants because of its relatively high absolute targeting efficiency. Finally, HDAdVs would be the best suited for manipulation of iPSCs for clinical applications, such as gene repair therapy of inherited disorders, because of the highly efficient and accurate gene targeting with minimal cytotoxicity at both transcriptionally active and inactive loci.

MATERIALS AND METHODS

Construction and preparation of HDAdVs. The human *HPRT1*-targeting HDAdV was described previously.¹⁶ To construct the human *HB9*-EGFP knock-in HDAdV, 5' and 3' homologous arms were amplified from KhES-1 genomic DNA by PCR. The ATG start codon of EGFP gene was fused in-frame with the ATG of *HB9* gene. The fragments were then introduced into the pBluescriptII-based vector plasmid encoding the PGK-neo-pA cassette which was sandwiched by *loxP* sites and then the whole targeting cassette was subcloned into an HDAdV plasmid. To generate the human *KU80*, *LIG1*, and *LIG3* gene-targeting vectors, BAC clones containing the human *KU80*, *LIG1*, and *LIG3* loci (BACPAC resources, Oakland, CA) were modified by using the RED/ET recombination technique.³⁵ The *loxP71*-PGK-EM7-neo-bpA-*loxP66* cassette was inserted into the target sites on these BAC clones (Figures 2a and 3a,b). Subsequently, a total of 22–23 kb of homologies including the marker cassette was subcloned into the HDAdV plasmid. A detailed description of these subclonings will be provided on request. The HDAdVs were propagated by serial passages on 293FLPc with addition of FL helper virus (kindly provided by Pedro Lowenstein)³⁶ and purified, as described previously.³⁷ Vector genome titers were determined by quantitative Southern analyses.

Culture of hESCs and hiPSCs. hESC lines, KhES-1 subline 1 and KhES-3,^{38,39} and hiPSC lines, 246H1 and 246G1 (kindly provided by Shinya Yamanaka),¹⁷ were maintained, as previously described.³⁹ 246H1 was derived from neonate fibroblast BJ cells with three factors (OCT3/4, SOX2, and KLF4). The hESC lines were used following the hESC research guidelines of the Japanese government.

Isolation of the gene-targeted clones. Clumps of hESCs and hiPSCs were infected with the HDAdVs at a multiplicity of infection of 1,000 vector genomes/cell. Electroporation of plasmid DNA was performed, as reported previously.³ G418 selection (50 μ g/ml; Nacalai tesque, Kyoto, Japan) was started 2 days after infection or electroporation. After 3 weeks, surviving colonies were transferred to 96-well plates and GANC selection (2 μ mol/l; Invitrogen, Carlsbad, CA) was started. For gene targeting at *HRPT1*, 6TG (10 mmol/l; Sigma-Aldrich, St Louis, MO) selection was applied after the G418 selection and the 6TG-resistant clones were analyzed. For other loci, G418/GANC double-resistant clones were characterized. Genomic DNA of drug-resistant clones was subject to screening by PCR using primers shown in Table 2 with LA Taq Hot Start Version (TAKARA, Kyoto, Japan) or PrimeSTAR GXL polymerase (TAKARA), following the manufacturer's protocol. To analyze the structure of *HB9*, *KU80*, *LIG1*, and *LIG3* loci, DNA was extracted from PCR-positive clones. The accuracy of gene targeting was carefully evaluated by the Southern analyses using restriction enzyme(s), which digested outside the homology arms and also inside the marker gene cassettes, but not within the homology arms. The 5' and 3' probes were located between the homology arms and the restriction enzyme sites. Restriction enzymes and probes for Southern hybridization are indicated in Figures 1a, 2a, 3a,b, and 4a. Additional ectopic integration of the vector is therefore expected to produce an extra band with the neo or GFP probe.

For the chromosomal analyses, gene knockout and knock-in hES clones were treated with 100 ng/ml colcemid (Invitrogen) for 2 hours, trypsinized, incubated in 0.075 mol/l KCl for 14 minutes, and fixed in Carnoy's fixative. The cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were analyzed by randomly counting 50 cells using the Ikaros Karyotyping System (MetaSystems, Waltham, MA).

Excision of the neomycin-resistant gene cassette in targeted clones. Clumps of hiPSCs were plated feeder-free and transfected with a pCAGGS-Cre plasmid, the CAG promoter-driven Cre gene, using FuGENE HD (Roche, Basel, Switzerland), according to the manufacturer's instructions. Six days later, the cells were sorted into single cells in the presence of Rho-associated coiled kinase (ROCK) inhibitor Y-27632 (10 μ mol/l; Wako,

Table 2 Primer sequences used in this study

Gene		Sequence (5' to 3')
Screening of the drug-resistant clone		
<i>HB9</i>	5' KO primer set	TGCGACTGTGTCCTGATGCCCGACTGTAAGCTG CGATGTTGTGGCGGATCTTGAAGTTCACCTTGA
	3' KO primer set	TCTTGTGATCAGGATGATCTGGACGAAGAG GGAGGACTTCTCAGAACACATTCCGCGCTCTTTG
<i>KU80</i>	5' KO primer set	CTCAGAAAGCACAGAACTAGGCATGAGAAG CTCTTCGTCCAGATCATCTGATCGACAAGA
	3' KO primer set	TCTTGTGATCAGGATGATCTGGACGAAGAG GGAGGCTTATATGAAGGCACGCATCAGATTCTCTAC
<i>LIG1</i>	5' KO primer set	AGGGATGAGAGGGCGGATGAAGGCATGAAT CTCTTCGTCCAGATCATCTGATCGACAAGA
	3' KO primer set	TCTTGTGATCAGGATGATCTGGACGAAGAG TTCTATCTTGTGCCAATCCAATGTGCCTGTAAAGC
<i>LIG3</i>	5' KO primer set	CAAAAACGCACTTTACTCTCCACCCTAACTAGCTGA CTCTTCGTCCAGATCATCTGATCGACAAGA
	3' KO primer set	TCTTGTGATCAGGATGATCTGGACGAAGAG CTGGGAGAACAAGATTATGTGTCCATCTGCTGTGA
Albumin for RT-PCR		GTTGCTCATCGGTTTAAAGATTTGGGAGAAGAA AGTTTGGGTTGTCATCTTTGTGTTGCAAG
Telomere-specific primer		TTAGGGTTAGGGTTAGGGTTAGGG

RT-PCR, reverse transcription-PCR.

Osaka, Japan) onto 96-well plates by using FACS Aria II (BD, Franklin Lakes, NJ). The removal of the *neo* cassette was confirmed by G418 sensitivity, PCR, and Southern hybridization, and the surviving colonies were used for motor neuron differentiation.

Motor neuron differentiation. Motor neuron induction was performed, as previously described with some modifications.⁴⁰ Briefly, dissociated iPSC colonies were plated onto culture dishes coated with poly-L-lysine/laminin (PLL/LM; Sigma-Aldrich) in N2B27 neural differentiation medium (1:1 mix of Dulbecco's modified Eagle medium/F12 supplemented with N2 and neurobasal medium supplemented with B27, all from Life Technologies, Carlsbad, CA), supplemented with mouse recombinant Noggin (100 ng/ml; R&D Systems, Minneapolis, MN) for 10 days. Subsequently, colonies were split into small clumps by treatment with Collagenase (200 U/ml; Life Technologies) with 1 mmol/l CaCl₂ and cultured on new poly-L-lysine/laminin-coated dishes with N2B27 supplemented with Noggin for 7 additional days. The cells were, then, dissociated by Accutase (Innovative Cell Technologies, San Diego, CA) and cultured on dishes coated with poly-L-lysine/laminin plus human plasma Fibronectin (PL/LM/FN) (Chemicon, Billerica, MA) in N2B27 supplemented with 1 μmol/l of retinoic acid (RA; Sigma-Aldrich) for 4 days, and then cultured in N2B27 supplemented with retinoic acid and human recombinant sonic hedgehog (500 nmol/l; R&D Systems) for additional 7–9 days. The cells were again dissociated by Accutase and cultured on poly-L-lysine/laminin plus human plasma Fibronectin-coated dishes in N2B27 supplemented with brain-derived neurotrophic factor (10 ng/ml; R&D Systems), glial cell-derived neurotrophic factor (10 ng/ml; R&D Systems), and neurotrophin-3 (10 ng/ml; R&D Systems) for another 5 days. Finally, the cells were fixed with 4% paraformaldehyde and subjected to direct visualization of EGFP and immunocytochemistry of HB9 protein, as previously described.⁴⁰

Quantitative RT-PCR assay and western blot assay. RNA extraction and cDNA synthesis for quantitative RT-PCR were carried out as previously described.¹⁶ The ABI PRISM 7000 sequence detection system (Applied Biosystems, Carlsbad, CA) was used for quantitative RT-PCR. Predesigned primers and probe sets for *KU80* (Hs00221707_m1), *LIG1* (Hs01553527_m1), *LIG3* (Hs00242692_m1), and *GAPDH* (Hs99999905_m1) were obtained from Applied Biosystems. Western blot assays were performed with anti-*KU80* (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-*LIG1* (10H5; Abcam, Cambridge, MA), anti-*LIG3* (1F3; Abcam), or anti-α-tubulin (DM1A; Santa Cruz Biotechnology) antibodies. The membranes were subsequently probed with a horseradish peroxidase-conjugated secondary antibody (anti-goat IgG-HRP or anti-mouse IgG-HRP; Santa Cruz Biotechnology) and developed with ECL PLUS (GE Healthcare, Piscataway, NJ).

Analyses of the stem cell markers and pluripotency. Expression of alkaline phosphatase and surface markers, such as SSEA-4, TRA-1-60, and TRA-1-81, was examined, as previously described.¹⁶ Formation of embryoid bodies, RNA extraction, and cDNA synthesis were previously described.¹⁶ The sequence of *Albumin*-specific primers are shown in **Table 2**. The sequences of other primers were previously described.¹⁶ Formation of teratoma and hematoxylin and eosin staining were performed with *HB9*-EGFP knock-in hESC and hiPSC clones, as previously described.³⁹ Animal protocols were approved by the Institutional Board on Animal Care.

SUPPLEMENTARY MATERIAL

Figure S1. Examples of Southern analyses.

Figure S2. Karyotype analyses.

Figure S3. Motor neurons differentiated from the *HB9*-EGFP knock-in hESC clone.

Table S1. The number of colonies in which the selection marker was excised.

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