Reports

A system for the measurement of gene targeting efficiency in human cell lines using an antibiotic resistance–GFP fusion gene

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Gene targeting in a broad range of human somatic cell lines has been hampered by inefficient homologous recombination. To improve this technology and facilitate its widespread application, it is critical to first have a robust and efficient research system for measuring gene targeting efficiency. Here, using a fusion gene consisting of hygromycin B phosphotransferase and 3'-truncated enhanced GFP (Hyg^R-5' EGFP) as a reporter gene, we created a molecular system monitoring the ratio of homologous to random integration (H/R ratio) of targeting vectors into the genome. Cell clones transduced with a reporter vector containing Hyg^R-5' EGFP were efficiently established from two human somatic cell lines. Established Hyg^R-5' EGFP reporter clones retained their capacity to monitor gene targeting efficiency for a longer duration than a conventional reporter system using an unfused 5' EGFP gene. With the Hyg^R-5' EGFP reporter system, we reproduced previous findings of gene targeting frequency being up-regulated by the use of an adeno-associated viral (AAV) backbone, a promoter-trap system, or a longer homology arm in a targeting vector, suggesting that this system accurately monitors H/R ratio. Thus, our Hyg^R-5' EGFP reporter system will assist in the development of an efficient AAV-based gene targeting technology.

Gene targeting in human somatic cells is a powerful technology allowing for the biological analyses of human gene function in physiological settings. Gene targeting is achieved by homologous recombination (HR) between the endogenous gene loci and targeting vectors introduced into cells. The use of gene-targeted human cell systems is particularly advantageous for the study of genetic changes leading to human diseases such as cancer (1,2). These cell systems are also expected to serve as a platform for compound screening and thereby facilitate the development and validation of molecularly targeted therapies (3,4).

Although gene targeting in human cells has improved significantly (5–9), the execution of gene targeting in human somatic cell lines continues to be labor-intensive and difficult to achieve depending on the genomic loci targeted and the cell lines used. This is probably because most somatic cells have low HR efficiency compared with embryonic stem cells as shown with murine myoblasts (10). To further improve this technology, it is critical

to first have a robust and efficient research system to measure gene targeting efficiency in human somatic cell lines. In practice, the ratio of homologous to random integration (H/R ratio) of targeting vectors should be closely monitored, as a high H/R ratio is critical for efficient gene targeting.

In previous studies, monitoring of gene targeting efficiency has been performed using vectors carrying reporter genes such as enhanced GFP (EGFP), neomycin phosphotransferase (Neo^R), and alkaline phosphatase genes that harbor intragenic inactivating mutations, deletions, or small insertions (5,11,12). Cells are first transduced with these reporter vectors and then with targeting vectors containing partial fragments of wild-type reporter genes that correct the inactivated reporter genes via HR and reconstitute intact reporter function. However, one concern with these reporter systems is unstable and insufficient expression of the reporter genes. Because reporter genes are generally driven by viral promoters, their expression tends to

diminish over time probably because of epigenetic changes within the viral promoters that arise during the establishment and maintenance of cell clones (13–15).

In this study, we sought to overcome this problem by using hygromycin B phosphotransferase (Hyg^R) fused with 3'-truncated EGFP (Hyg^R-5' EGFP) as a reporter gene. If the Hyg^R-5' EGFP gene is epigenetically silenced in a fraction of a reporter cell clone, this fraction will be eliminated by hygromycin selection. To evaluate the utility of this Hyg^R-5' EGFP reporter system, we assayed the H/R ratios of targeting vectors in human cell lines in several experimental settings.

Materials and methods

Vector construction

For the creation of the Hyg^R-5' EGFP reporter vector, the 5' portion of EGFP (500 bp) was amplified by PCR and ligated to an *Eco*RI-*Bam*HI site of pIRESneo3 (BD Biosciences, Franklin Lakes, NJ, USA). A *Bst*XI-*Xba*I

fragment containing a synthetic intron, internal ribosomal entry site (IRES), and Neo^R was then deleted from the resulting plasmid, and the remaining fragment was blunt-ended and self-ligated. This plasmid was then digested with *NheI* and *AgeI* and ligated to the PCR-amplified Hyg^R coding region with a Kozak consensus sequence (GCCACC) at its 5' terminus, so that the Hyg^R-5' EGFP was translated in frame. Finally, the 3' homology arm consisting of the internal portion of simian virus 40 (SV40) large T (1 kb) was PCR-amplified and inserted into a *BstZ171* site of the plasmid.

To construct ATG-less targeting vector (TV), pAAV-MCS (Agilent Technologies, Santa Clara, CA, USA) was digested with *NotI*, and the backbone containing two inverted terminal repeats was recovered. Multiple restriction enzyme sites, including the *NotI* and *XhoI* sites, were then incorporated into this backbone, destroying the original *NotI* sites and thus generating "modified pAAV-MCS." Meanwhile, an EGFP coding region without an authentic start codon was PCR-amplified and inserted into an *XbaI* site of pSEPT (a gift from Dr. Fred Bunz) (16) in the forward direction. The resulting plasmid was cut with *NotI* and *XhoI*, and the isolated *NotI*-*XhoI* fragment containing ATG-less EGFP and a promoter-trap assembly was ligated to a *NotI*-*XhoI* site of the modified pAAV-MCS. The resulting plasmid was finally digested with *XhoI* and ligated to an *XhoI* fragment from the Hyg^R-5' EGFP reporter consisting of the internal portion of SV40 large T (i.e., the 3' homology arm of the Hyg^R-5' EGFP reporter).

For the construction of SV40p TV, the bovine growth hormone polyadenylation site [BGH poly(A)] derived from pcDNA3.1 (Life Technologies, Carlsbad, CA, USA) was first inserted at the *EcoRV* site of plasmid 5A (a gift from Dr. Ben H. Park), a platform vector to construct targeting vectors carrying a Neo^R gene expressed by an SV40 early promoter (17). The resulting plasmid containing BGH poly(A) in the forward direction was cleaved with *NheI* and *XmnI* to remove the backbone of the plasmid. The recovered fragment was then blunt-ended and ligated in the forward direction to a blunt-ended *SpeI*-*EcoRV* fragment from ATG-less TV containing a backbone and both of the homology arms.

For 3' EGFP TV, a fragment between two *XbaI* sites in ATG-less TV (i.e., the EGFP coding region without an authentic start codon) was replaced with a PCR-amplified 3' partial EGFP coding region (606 bp).

To produce the 5' EGFP reporter vector, the 5' portion of EGFP (500 bp) with a cytomegalovirus promoter was recovered as a *SpeI* fragment derived from pEGFPx2 (a gift from Dr. Ben H. Park) and inserted into

Table 1. Establishment of reporter clones.

Reporter gene	Cell line	Single cell clones screened	Functional clones established
Hyg ^R -5' EGFP	HCT116	6	1 (16.7%)
	DLD-1	6	1 (16.7%)
	Total	12	2 (16.7%) ^a
5' EGFP	HCT116	69	2 (2.9%) ^a

^aNo statistical significance, Fisher's exact test.
Hyg^R-5' EGFP, a fusion gene consisting of hygromycin B phosphotransferase and 3'-truncated enhanced GFP; 5' EGFP, 3'-truncated enhanced GFP.

Figure 1. Representation of the reporter and targeting vectors. Vector names are denoted to the right (a reporter vector) or left (targeting vectors) of the diagrams. A filled rectangle indicates the location of a probe used for Southern blot analysis in Figure 2C. *BglII* and *BamHI* sites on the Hyg^R-5' EGFP reporter vector that were cleaved during Southern blot analysis are marked in the diagram. Hyg^R, hygromycin resistance gene; TV, targeting vector; CMVp, cytomegalovirus promoter; IVS, synthetic intron; IRES, internal ribosomal entry site; Neo^R, neomycin resistance gene; SV40p, simian virus 40 promoter; pA, poly(A) sites from SV40 early mRNA (Hyg^R-5' EGFP reporter, ATG-less TV, and 3' EGFP TV), the bovine growth hormone gene (SV40p TV, left), or a 49-bp-long synthetic poly(A) signal derived from pCI-neo (SV40p TV, right). Solid lines indicate vector sequences other than those specified above. Dotted lines indicate homologous regions between reporter and targeting vectors. For details on partial EGFP genes, see vector construction in the Materials and methods section. The diagrams are not drawn to scale.

pSEPT in the forward direction. To introduce a 3' homology arm, the resulting plasmid was cleaved with *XhoI* and then ligated to the internal portion of SV40 large T excised from the Hyg^R-5' EGFP reporter vector. After transfection of the resulting plasmid into cells, stable transfectants were infected with an adenovirus encoding Cre, and a subclone undergoing Cre-loxP recombination was isolated from each stable clone; the H/R ratio was then measured by infection with ATG-less TV. Throughout the construction of all plasmids described above, fragments amplified by PCR were confirmed by DNA sequencing after cloning into plasmids. The sequences of the plasmids have been deposited in GenBank (accession numbers: Hyg^R-5' EGFP reporter, JQ394982; ATG-less TV, JQ394983; SV40p TV, JQ394984; 3' EGFP TV, JQ394985; and 5' EGFP reporter, JQ394986).

Transfection and infection

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Chemicals, Osaka, Japan) supplemented with 5% fetal bovine serum. Transfection of plasmids into cells was performed using TransIT-LT1 (Mirus Bio, Madison, WI, USA) as per the manufacturer's instructions. Adeno-associated viral

(AAV) particles (serotype 2) were produced by transfection of 293T cells in 75 cm² flasks with 7.5 μg AAV-based targeting plasmids and the AAV Helper-Free system (Agilent Technologies). The copy numbers of the AAV particles were determined by real-time PCR performed with StepOnePlus (Life Technologies), using the solutions of the AAV-based targeting plasmids as controls. The copy numbers of the AAV-based targeting plasmids in the control samples were calculated using their molecular weights and concentrations measured by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA).

Throughout the study, cells were infected with AAV particles as previously described (7) at a multiplicity of infection of 1×10^4 . Selection with G418 (Life Technologies) was carried out at concentrations of 0.4 and 1 mg/mL for the HCT116 and DLD-1 cell lines, respectively. In addition, 0.2 and 0.25 mg/mL hygromycin (Wako Chemicals) were used for the selection of HCT116 and DLD-1 cells, respectively. For the assay of the H/R ratio of the targeting vectors, reporter cell clones were infected with AAV-based targeting vectors in 75-cm² flasks, selected with G418 for 1–2 weeks, trypsinized, suspended in phosphate-buffered saline

Figure 2. The Hyg^R-5' EGFP reporter system for monitoring gene targeting efficiency in human cell lines. (A) Representative flow cytometry results. The reporter and targeting vectors shown on the top were sequentially transduced into cells, and GFP-positive ratios were determined by fluorescence flow cytometry. Hereafter, AAV infection of the cells was performed at a multiplicity of infections of 1×10^4 . FL1A (x-axis) and FL2A (y-axis) represent GFP signals and autofluorescence from cells, respectively. The GFP-positive ratios for the respective experiments are noted in the scatter plots. (B) Graphical display of all of the results ($n = 3$; mean $\pm$ SEM). (C) Southern blot analysis investigating the copy number of the Hyg^R-5' EGFP reporter vector within the genome of each reporter clone. gDNA was extracted from parental DLD-1 (D) and HCT116 (H) cell lines (parent) as well as from Hyg^R-5' EGFP reporter clones (reporter) established from the parental cells, digested with either *Bam*HI or *Bgl*II, as noted below the gel image, and analyzed with a probe located on the Hyg^R coding sequence as depicted in Figure 1. Single bands detectable in Hyg^R-5' EGFP reporter clones are marked with white arrowheads. The positions of DNA size standards are shown to the right. Hyg^R, hygromycin resistance gene; TV, targeting vector.

(PBS), and analyzed with a FACSCanto II flow cytometer (BD Biosciences). Cell sorting was performed with a FACS Vantage SEM instrument (BD Biosciences).

Polymerase chain reaction

PCR was performed using Platinum *Taq* (Life Technologies) or *Pwo* DNA Polymerase (Roche, Basel, Switzerland) and a GeneAmp 9700 thermal cycler (Life Technologies). Genomic DNA (gDNA) for PCR templates was extracted using PureLink Genomic DNA Mini kit (Life Technologies) according to the manufacturer's instructions. To verify homologous integration of the targeting vector into the genome, PCR was performed using the following oligonucleotide primers: F1, 5'-TGTGTAGAAAGTACTCGCCGA-3'; R1, 5'-TCCAGCAGGACCATGTGATC-3'; F2, 5'-ACCATCTTCTTCAAGGACGA-3'; and R2, 5'-AGTACTCACCCCAACAGCTG-3'.

Southern blot analysis

For Southern blot analyses, gDNA was extracted using standard phenol/chloroform extraction and ethanol precipitation. Five micrograms gDNA were digested with the appropriate restriction enzymes, fractionated on 0.7% agarose gels, and blotted onto Hybond-N+ membrane (GE Healthcare, Piscataway, NJ, USA). Probes were labeled using AlkPhos Direct Labeling and Detection System with CDP-Star kit (GE Healthcare) and used for hybridization.

Statistical analyses

Statistical analyses were performed using Intercooled Stata (Stata, College Station, TX, USA). The efficiency of establishing reporter clones was analyzed with Fisher's exact test. Two-way factorial analysis of variance was applied for the comparison of plasmid-based versus AAV-based targeting vectors and ATG-less TV versus SV40p TV after log trans-

formation of the data. A paired *t*-test was used to compare the short and long 5' arms.

Results and discussion

We initially constructed a reporter vector, the Hyg^R-5' EGFP reporter, carrying a fusion gene consisting of full-length Hyg^R (5' side) and a 5' portion of EGFP (500 bp in length; 3' side) fused to each other in a single frame (Figure 1). We also constructed a targeting vector, ATG-less TV (Figure 1), containing an EGFP gene without an authentic start codon to reconstitute functional EGFP via HR with the Hyg^R-5' EGFP fusion gene in the reporter vector. ATG-less TV uses a promoter-trap strategy for efficient gene targeting (18,19) and thus has no promoter to drive Neo^R expression. In addition, ATG-less TV is constructed with an AAV backbone, because AAV-based targeting vectors achieve more than 1000-fold higher rates of gene targeting than conventional plasmid-based targeting vectors (5).

When cells are stably transduced with the Hyg^R-5' EGFP reporter vector and the ATG-less TV, gene-targeting events are detectable by a green fluorescent signal. Because the H/R ratio of targeting vectors is one of the most critical determinants of the difficulty of gene targeting, we addressed the ability of this system to assay H/R ratios. We first transduced the Hyg^R-5' EGFP reporter vector into the HCT116 and DLD-1 colon cancer cell lines, and isolated single cell clones (hereafter referred to as reporter clones). The resulting six reporter clones from HCT116 and six from DLD-1 were then infected with ATG-less TV and selected with G418. Fluorescence flow cytometric analyses of the resulting bulk cell populations exhibited subsets of GFP-positive cells in one reporter clone from each cell line (Figure 2, A and B, and Table 1). These results strongly suggested that homologous regions within the two vectors were recombined in subsets of cells in these clones, leading to the production of functional GFP. We also confirmed the absence of appreciable GFP-positive cell populations within parental HCT116 and DLD-1 cells as well as in cells transduced with either the Hyg^R-5' EGFP reporter or ATG-less TV alone. Southern blot analysis of a functional reporter clone from each of these cell lines demonstrated that these reporter clones are likely to harbor single copies of the Hyg^R-5' EGFP reporter vector in the genome (Figure 2C).

In the other five reporter clones for each cell line, no GFP-positive cell populations were detected by flow cytometry after infection with ATG-less TV. Although the reason for this is unclear, it may be possible that the location of the Hyg^R-5' EGFP reporter vector within the genome affects the efficiency of HR

with ATG-less TV. Another possibility is that all or part of the 3' arm of the Hyg^R-5' EGFP reporter vector may be subjected to terminal deletion upon integration into the genome, which precludes gene targeting by ATG-less TV.

We next carried out cell sorting of an HCT116-derived reporter clone transduced with ATG-less TV and isolated GFP-positive and -negative populations. A total of 15 single cell clones were established from the respective populations and analyzed by PCR to determine whether GFP-positive cells had truly undergone gene targeting by ATG-less TV. PCR over the 5' arm of ATG-less TV (F1–R1; Figure 3, A and B) indicated that gene targeting was achieved in at least 14 of the 15 single cell clones isolated from the GFP-positive cell population, whereas none of the clones from the GFP-negative population underwent gene targeting. We also confirmed the reconstitution of a full-length Hyg^R-EGFP gene in a few GFP-positive single cell clones by DNA sequencing (data not shown). In addition, we carried out Southern blot analyses of several GFP-positive and -negative single cell clones derived from the HCT116 and DLD-1 cell lines, and further confirmed that ATG-less TV was homologously recombined with the Hyg^R-5' EGFP reporter vector within the genome of GFP-positive cells (Figure 3C). These results collectively indicate that GFP positivity in the Hyg^R-5' EGFP reporter system represents gene targeting events.

To determine the accuracy of the Hyg^R-5' EGFP reporter system for monitoring gene targeting efficiency, we subsequently investigated whether this reporter system could reproduce previous findings of gene targeting efficiency achieved with various types of targeting vectors. We first infected (as an AAV-based targeting vector) or transfected (as a plasmid-based targeting vector) HCT116 and DLD-1-derived Hyg^R-5' EGFP reporter clones with ATG-less TV and then selected the cells with G418. Flow cytometric analyses demonstrated that AAV-based ATG-less TV infection achieved an average of >100-fold gene targeting efficiency compared with that of the plasmid version of ATG-less TV (Figure 4A), reproducing previous results (5,6,16). We also addressed the impact of the promoter-trap strategy on gene targeting efficiency in this system. To this end, we constructed an AAV-based targeting vector similar to ATG-less TV, except that it lacked a synthetic intron and IRES, containing a poly(A) site and an SV40 promoter to drive Neo^R instead, thus no longer supporting the promoter-trap strategy (SV40p TV; Figure 1). Fluorescence flow cytometric analyses demonstrated that the infection of Hyg^R-5' EGFP reporter cell clones with ATG-less TV achieved, on average, 40-fold greater gene

Figure 3. Confirmatory assays demonstrating that GFP-positive clones were generated by gene targeting. (A) The Hyg^R-5' EGFP reporter vector, ATG-less TV, and a product of homologous recombination between these vectors. F1, F2, R1, and R2 indicate the location of the primers used in the PCR shown in panel B. Filled rectangles indicate the location of probes on Hyg^R (a) and partial SV40 large T (b) genes used in the Southern blot analyses shown in panel C. Restriction enzyme sites were abbreviated as follows and marked on the diagrams: S, SacI; P, PvuII; E, EarI. Arrows at the bottom show the distances between the restriction enzyme sites in kilobase pairs. The diagrams are not drawn to scale. (B) PCR over the 5' homology arm of ATG-less TV. The HCT116-derived Hyg^R-5' EGFP reporter cell clones infected with ATG-less TV were sorted into GFP-positive and -negative populations by flow cytometry. A total of 15 single cell clones from each of the populations was expanded and analyzed by PCR. The size of the PCR amplicons is denoted to the right. Note that a faint band seen in the 11th lane from the left on the F1–F2 gel image was determined as being marginally positive. PCR primers F1, R1, F2, and R2 are depicted in panel A. No targeting: a reporter clone not infected with the targeting vector. (C) Southern blot analyses of GFP-positive and -negative single cell clones. Several DLD-1–derived and HCT116–derived single cell clones were established as in panel B, and gDNA from these clones was digested with the restriction enzymes indicated below the gel images. Probes (a) and (b) depicted in panel A were used for Southern hybridization with SacI/PvuII-digested gDNA and SacI/EarI-digested gDNA, respectively. Note that probing for Hyg^R and SV40 large T sequences leads to the detection of one and two bands, respectively, in Hyg^R-5' EGFP reporter clones with random integration of ATG-less TV into the genome. The positions of the DNA size standards are shown to the right of each image. Parent, parental DLD-1 or HCT116 cell line; reporter, Hyg^R-5' EGFP reporter cell clones not infected with the targeting vector. Hyg^R, hygromycin resistance gene; TV, targeting vector; CMVp, cytomegalovirus promoter; IVS, synthetic intron; IRES, internal ribosomal entry site; Neo^R, neomycin resistance gene; pA, poly(A) sites.

targeting efficiency than SV40p TV (Figure 4B), which is similar to the results reported by previous studies (6,16). Increasing homology length is also likely to increase targeting rates (11). We thus shortened the 5' homology arm of ATG-less TV and examined its impact on gene targeting efficiency. The 5' homology arm of ATG-less TV is 719 bp in length,

and a 113-bp fragment at its 5' terminus was deleted to create another targeting vector, 3' EGFP TV (Figure 1). This truncation resulted in a reproducible decrease in gene targeting efficiency, although our results did not reach statistical significance (Figure 4C). Overall, our Hyg^R-5' EGFP reporter system successfully reproduced the impact of targeting vector

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Figure 4. The Hyg^R-5' EGFP reporter system monitors the efficiency of gene targeting performed by various targeting vectors. The Hyg^R-5' EGFP reporter clones were transduced with the targeting vectors denoted at the bottom of each panel, selected with G418, and analyzed by fluorescence flow cytometry. (A) Reporter clones were either transfected (as a plasmid) or infected (as an AAV) with ATG-less TV ($n = 3$; mean $\pm$ SEM). (B) Reporter clones were infected with AAV-based targeting vectors with (ATG-less TV) or without (SV40p TV) a promoter-trap system ($n = 3$; mean $\pm$ SEM). (C) A HCT116-derived reporter clone was infected with targeting vectors containing 5' homology arms that were 606 bp (3' EGFP TV; short 5' arm) or 719 bp (ATG-less TV; long 5' arm) in length. For panels A–C, uninfected reporter clones were used as controls. Asterisks indicate a GFP-positive ratio of 0%.

designs on gene targeting efficiency described in previous studies.

To clarify the advantage of the use of our Hyg^R-5' EGFP reporter system in monitoring gene targeting efficiency, we next compared the efficiency of generating reporter clones in our Hyg^R-5' EGFP system with that of a conventional reporter system that uses an unfused 5' EGFP gene as a reporter (5' EGFP reporter). We first isolated multiple HCT116-derived cell clones transduced with the 5' EGFP reporter vector and infected the clones with ATG-less TV. The resulting bulk population of G418-resistant cells from each 5' EGFP reporter clone was then analyzed by fluorescence flow cytometry, similar to that for the Hyg^R-5' EGFP reporter clones. It appeared that functional reporter clones were obtained with higher efficiency by the Hyg^R-5' EGFP reporter system than that with the 5' EGFP reporter system (Table 1), although the difference did not reach statistical significance presumably because of the limited number of cell clones analyzed. Southern blot analysis demonstrated that the two functional 5' EGFP reporter clones are likely to carry single 5' EGFP reporter vectors within the genome (data not shown).

We also addressed the ability of reporter cell clones in the Hyg^R-5' EGFP and 5' EGFP reporter systems to monitor gene targeting efficiency over time. Reporter clones bearing functional Hyg^R-5' EGFP or 5' EGFP reporter genes were maintained in culture for long durations (no less than 4 weeks), infected with ATG-less TV, selected with G418, and then analyzed by fluorescence flow cytometry. GFP-positive ratios in individual reporter clones were then compared with those obtained before the above mentioned long-term culture. We found that GFP-positive ratios before and after the long-term culture were equivalent in the Hyg^R-5' EGFP reporter clones; however, in 5' EGFP reporter clones, GFP-positive ratios were dramatically decreased after the long-term

culture (Figure 5). These data demonstrated that our Hyg^R-5' EGFP reporter system provides a prolonged capacity to monitor gene targeting efficiency relative to a conventional reporter system.

In this study, we developed a molecular system to monitor gene targeting efficiency in human somatic cell lines. This system uses the Hyg^R-EGFP fusion gene for a dual purpose, namely, antibiotic selection as well as fluorescence-based monitoring of gene targeting events. When a reporter gene is expressed independent of an antibiotic resistance gene, as in conventional reporter vectors, cell clones transduced with such vectors rarely serve as potent reporter systems (Table 1). Moreover, reporter cell clones bearing such reporter vectors are prone to lose their function over time during cell culture (Figure 5). In contrast, the use of the Hyg^R-EGFP fusion gene allows the establishment of potent reporter clones in a less labor-intensive fashion, and the resultant reporter clones retain their function over a long duration. Indeed, the Hyg^R-5' EGFP reporter clones were functional throughout the study period of more than 6 months.

Another strategy permitting the acquisition of potent reporter clones in an efficient fashion may be the use of IRES to join a selection marker gene and a reporter gene instead of directly fusing them (12). By maintaining reporter clones in a selective condition, this type of reporter system may also ensure robust, prolonged expression of reporter genes. However, cistrons joined by IRES do not always produce an equivalent level of proteins. It has been shown that the amounts of two proteins produced from a transcript containing IRES vary in a position-dependent fashion (20), as well as by host cell type (21,22), presumably because translation downstream of IRES is modulated by multiple regulatory factors (23,24). In contrast, a fusion protein in our system secures the production of exactly the same amount of antibiotic resis-

Figure 5. Use of the Hyg^R-5' EGFP gene ensures robust and prolonged reporter activity. The gene targeting efficiency of the reporter clones denoted at the bottom of the figure was assayed twice with an interval of no less than 4 weeks. In each assay, reporter clones were transduced with ATG-less TV, selected with G418, and analyzed by flow cytometry to quantify GFP signals. Analyses included one each of the HCT116 and DLD-1-derived Hyg^R-5' EGFP reporter clones as well as two HCT116-derived 5' EGFP reporter clones. The second assays were performed on the 82nd (HCT116, Hyg^R-5' EGFP), 69th (DLD-1, Hyg^R-5' EGFP), 28th (HCT116#1, 5' EGFP), and 36th (HCT116#2, 5' EGFP) day after the first assays. The first assays for the 5' EGFP reporter clones were performed without multiplication and thus have no error bars. The other assays were performed in triplicate (mean $\pm$ SEM).

tance and reporter proteins, thus offering more stable activity as a reporter.

Another strategy to prevent attenuation of reporter gene function is the use of an endogenous gene as a reporter. One of the endogenous genes most commonly used for this purpose is the hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene on the X chromosome. In cells bearing a single *HPRT1*, such as diploid cells of male origin, the disruption of single *HPRT1* alleles results in the loss of hypoxanthine phosphoribosyltransferase, which is selectable with an anti-metabolic nucleoside analog 6-thioguanine (6-TG) (5,25). Furthermore, targeted correction of mutant *HPRT1* can be detected by culturing cells with hypoxanthine-aminopterin-thymidine (HAT) medium (25,26). As a drawback, however, this system cannot be utilized with cells bearing multiple X chromosomes, such as female cells and majority of aneuploid cells. In addition, cells exhibiting the microsatellite instability (MIN) phenotype are generally resistant to 6-TG (27) and are thus not eligible for *HPRT1* targeting followed by 6-TG selection. In contrast, our reporter system is suitable for virtually any type of cell of male or female origin, including those with a MIN phenotype. Furthermore, our system determines gene targeting efficiency through rapid, easily performed high-throughput procedures with the aid of flow cytometry, unlike the *HPRT1*-based system in which gene targeting efficiency is estimated by counting colonies on tissue culture plates after incubation of the cells with and without 6-TG.

Besides determining the H/R ratio of targeting vectors, the Hyg^R-5' EGFP system permits estimation of the absolute gene targeting frequency. This is accomplished by counting colonies formed on tissue culture plates after antibiotic selection in parallel with flow cytometric analyses of antibiotic-resistant cells. The absolute gene targeting frequency can be estimated by multiplying the number of colonies on the plate by the GFP-positive ratio determined by flow cytometry and then dividing the resulting number by the number of cells prepared for infection of the targeting vector.

Although gene targeting in human somatic cells can be achieved by several different methodologies (12,28–30), AAV-mediated gene targeting has an advantage in that it does not actively introduce DNA double-strand breaks, thus likely generating fewer undesired alterations in the genome. Because the technology of AAV-mediated gene targeting is simple, cost-sensitive, publicly available, and capable of modifying human somatic cell genomes with decent efficiency, it continues to be an important alternative methodology for genome editing in human cell lines. Indeed, AAV-mediated gene targeting has been utilized in many recent studies modifying genomes in human cell lines with the aim of gene functional analyses (4,17,31–38). As shown in this study, our Hyg^R-5' EGFP reporter system enables robust monitoring of the efficiency of AAV-mediated gene targeting in human somatic cells. This reporter system will thus prove helpful in improving the design

of AAV-based targeting vectors and optimizing experimental conditions for gene targeting. It may also assist in screening for genes and drugs that affect the incidence of HR, potentially leading to further improvements in gene targeting technology. Such technical improvements will eventually result in the generation of highly efficient gene targeting systems readily applicable to various types of human cells.

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Competing interests

The authors declare no competing interests.

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