

Novel reporter systems for facile evaluation of I-SceI-mediated genome editing

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ABSTRACT

Two major limitations to achieve efficient homing endonuclease-stimulated gene correction using retroviral vectors are low frequency of gene targeting and random integration of the targeting vectors. To overcome these issues, we developed a reporter system for quick and facile testing of novel strategies to promote the selection of cells that undergo targeted gene repair and to minimize the persistence of random integrations and non-homologous end-joining events. In this system, the gene target has an I-SceI site upstream of an EGFP reporter; and the repair template includes a non-functional EGFP gene, the positive selection transgene MGMP140K tagged with mCherry, and the inducible Caspase-9 suicide gene. Using this dual fluorescent reporter system it is possible to detect properly targeted integration. Furthermore, this reporter system provides an efficient approach to enrich for gene correction events and to deplete events produced by random integration. We have also developed a second reporter system containing MGMP140K in the integrated target locus, which allows for selection of primary cells with the integrated gene target after transplantation. This system is particularly useful for testing repair strategies in primary hematopoietic stem cells. Thus, our reporter systems should allow for more efficient gene correction with less unwanted off target effects.

INTRODUCTION

Traditional retrovirus-based gene therapy approaches are based on semi-random provirus integration of therapeutic

transgenes in target cells. Clinical trials utilizing this type of technology for the treatment of patients with monogenic inherited disorders, namely X-linked severe combined immunodeficiency (X-SCID) (1), Adenosine deaminase deficiency severe combined immunodeficiency (ADA-SCID) (2) and X-linked Chronic granulomatous disease (X-CGD) (3) have produced promising results. However, the occurrence of several adverse events as consequence of insertional mutagenesis in the X-SCID trials (4,5) in addition to the clonal expansion observed in the CGD patients (3) have exposed the limitations of this technology. Recently, promising alternatives have emerged with the potential to circumvent this safety issue by using engineered homing endonucleases (6,7) or zinc-finger nucleases (8) to direct the processes of gene correction and targeted gene insertion. This approach allows for targeted insertion of a therapeutic gene at a predetermined locus or the modification of a disease gene in its native locus. To date, homing endonucleases and zinc-finger nucleases have been used in numerous species to generate site-specific DNA double-strand breaks that, if repaired by homologous recombination, lead to targeted gene correction (8,9).

LAGLIDADG homing endonucleases are ideal for genome editing because they recognize very long DNA sequences, between 14 and 40 bp, and although they tolerate some sequence degeneracy, the frequency of random cleavage is very low (10). Homing endonucleases have been used successfully in numerous studies of gene correction employing *in vitro* and *in vivo* models (11,12). In light of these promising results, significant efforts are currently being made to engineer homing endonucleases with reduced toxicity (13), and to modify their specificity to efficiently cleave disease genes loci that may be corrected through this approach (6,14).

Several monogenic disorders found in dogs such as X-linked severe combined immunodeficiency (15), Duchenne muscular dystrophy (16) and hemophilia (17)

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among others, are caused by orthologs of human genes. Thus, the dog represents a highly relevant pre-clinical animal model for the study of targeted gene correction of genetic disorders. Nevertheless, proof-of-concept studies in the canine model are required to validate the feasibility of this approach to gene therapy.

We have previously shown that expression of methylguanine methyltransferase P140K (MGMT_{P140K}), a mutant enzyme that in contrast to wild-type MGMT is not inhibited by O₆-benzylguanine (O₆BG), allows for efficient and stable selection of gene-modified hematopoietic stem cells in both canine and non-human primate models (18,19). Here we took advantage of this selection mechanism to test strategies to improve the efficiency of targeted genome editing approaches. In one of the reporter systems we describe here, MGMT_{P140K} expression mediates the positive selection of cells that have undergone targeted gene correction. This approach is useful when the gene-corrected cells do not have a selective advantage relative to the non-corrected counterparts. Additionally, we included a negative selection marker, an inducible caspase-9 gene (20), to facilitate the elimination of integration events that are not generated by homologous recombination, i.e. repair substrate captured at the site of I-SceI cleavage or randomly integrated. This dual-selection approach represents a novel mechanism to facilitate the enrichment of cells that have undergone site-directed gene correction.

In the other reporter system we developed, MGMT_{P140K} expression allows for the selection of the cells with the integrated target locus prior to targeting studies. This type of system would be useful for the *in vitro* and *in vivo* characterization of gene repair strategies in the context of genetic disease where all of the cells would harbor the genomic loci for targeted integration and relatively low levels of gene correction would lead to therapeutic benefit (i.e. X-SCID).

MATERIALS AND METHODS

Vector production

To generate integrating lentivirus vectors, HEK 293T cells were transiently transfected with the VSV-G expression plasmid pMD2.G (kindly provided by Didier Trono—École Polytechnique Fédérale de Lausanne, Switzerland—, and Luigi Naldini—Fondazione San Raffaele, Milan, Italy), the packaging plasmid pCMV8.74 (kindly provided by Didier Trono and Luigi Naldini) and the vector of interest following a protocol previously described (21) with two modifications. Specifically, PEI-mediated gene transfer was used instead of Calcium phosphate, and 6 µg of pMD2.G were utilized instead of 9.5 µg. Viral particles were concentrated by overnight centrifugation at 7200g, and the viral titer was determined by infecting human osteosarcoma cells (HT1080) with serial dilutions of the viral preparation followed by either Q-PCR or detection of the fluorescent transgene by FACS.

To produce integrase-deficient lentiviruses (IDLVs), HEK 293T cells were transiently transfected as described

before (22), with the vector, the VSV-G expression plasmid pMD2.G and the packaging plasmid psPax2 (Addgene# 12260), which contains a point mutation in the integrase gene (D64V). The virus preparation was then concentrated at 8500g overnight. The viral titer was determined by p24 ELISA using Alliance p24 Antigen ELISA kit (Perkin-Elmer) according to the manufacturer's protocol.

Quantitative PCR

The virus titer of the target vector N-stopEGFP target was determined by quantitative PCR as described previously (18).

Gene correction on D-17 cells with the integrated I-EGFP target

A lentiviral vector encoding the I-EGFP gene target was used to transduce D-17 cells (obtained from ATCC) at a low multiplicity of infection of 0.02. The cells were subject to two rounds of FACS sort until >95% of the cells were EGFP positive. Subsequently, 1×10^5 D-17 cells with the integrated target were plated one day before transduction IDLVs encoding the repair substrate, Δ 3-19EGFP and I-SceI driven by a SFFV promoter. The day of the transduction, the culture medium was replaced and supplemented with 8 µg/ml protamine sulfate, and 100 ng/ml of HIV-1 p24 of each IDLV were added to the cells.

For the negative selection of random integrants, the cells were treated three times with 1 µM of synthetic dimerizer AP20187 (kindly provided by Ariad Pharmaceuticals, Inc, Cambridge MA); treatment with vehicle alone, i.e. 100% ethanol, was performed as control. The cells were allowed to recover and then treated three times with 50 µM O₆-benzylguanine (O₆BG) and 200 µM 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). The phenotypes of the cells were evaluated via FACS. The following primers were used to amplify the gene target: hPGKf2-5'-GTG TTC CGC ATT CTG CAA GCC TC-3' and EGFPPr-5'-CTT GTG GCC GTT TAC GTC GCC G-3' and the amplicons were sequenced as described above. To evaluate the presence of the iCaspase9 transgene and differentiate homologous recombination from NHEJ, the following primers were utilized: mCherry[nucleotide 639]-5'-CAC CAT CGT GGA ACA GTA CG-3' and SIN-LTR[nucleotide 26]-5'-TCG TTG GGA GTG AAT TAG CC-3' with 200 ng of genomic DNA as template.

Gene correction on D-17 cells with the integrated N-stopEGFP target

A lentiviral vector encoding the N-stopEGFP gene target was used to transduce D-17 cells (canine osteosarcoma) at a multiplicity of infection of 0.9. The transduced cells were subject to three rounds of treatment with 50 µM O₆BG and 200 µM BCNU in order select for cells with chromosomally integrated targets.

A total of 1×10^5 D-17 cells with the integrated target were plated one day before transduction with IDLVs expressing the repair substrate, C- Δ 14EGFP and I-SceI driven by a SFFV promoter. The day of the transduction,

the culture medium was replaced and supplemented with 8 μ g/ml protamine sulfate. Subsequently, various amounts of HIV-1 p24 of each IDLV were added to the cells and EGFP⁺ cells were quantified by FACS 48–72 h after transduction.

EGFP⁺ D-17 cells generated by gene correction were purified through FACS sorting to >90% EGFP⁺. Genomic DNA was extracted (Gentra Puregene Cell Kit, QIAGEN) and the gene target was amplified by PCR using the PhusionTM Flash High-Fidelity PCR Master Mix (Finnzymes) with the following primers: hPGK-forward 5'-CAC GTC GGC AGT CGG CTC CCT CG-3' and EGFP target-reverse 5'-CTC GTC CAT GCC GAG AGT GAT C-3'. The amplicon (799 bp) was subcloned into a pCR4-TOPO[®] vector (Invitrogen) following the manufacturer's instructions and then sequenced using standard procedures.

RESULTS

Gene correction of D-17 cells with the integrated I-EGFP target

As shown in Figure 1a, the gene target in this reporter system is referred to as I-EGFP, and it consists of a PGK promoter that drives the expression of an EGFP transgene that contains a recognition site for I-SceI 11-bp upstream of the EGFP start codon. The repair template, named Δ 3-19EGFP, consists of a PGK promoter that drives the expression of an mCherry-tagged MGMTP140K transgene, and a non-fluorescent mutant EGFP transgene that lacks 48 bp corresponding to aminoacids 3–19. This repair template contains regions of homology with the target locus in the PGK promoter and the EGFP gene that facilitate homologous recombination. Thus, if I-SceI-driven gene correction occurs, the EGFP positive cells that contain the integrated gene target become EGFP negative, mCherry positive and with the knock-in of MGMTP140K acquire resistance to treatment with O6BG and BCNU (Figure 1a). Importantly, capture of the repair template at the site of the I-SceI-mediated double-strand break (23) will acquire the same phenotype; however, because the repair template vector includes an inducible Caspase-9 transgene, these events can be eliminated through treatment with the Caspase-9 activating synthetic dimerizer AP20187. Also, if random integration of the repair template takes place, the cells become EGFP positive, mCherry positive, and are also sensitive to AP20187 due to the expression of the inducible Caspase-9 gene (Figure 1a).

To model the proposed dual-selection approach, a polyclonal culture of D-17 cells with stably integrated I-EGFP gene targets was transduced with two IDLVs, one encoding the Δ 3-19EGFP repair template and the other encoding I-SceI as indicated in Figure 1a. The anticipated results of the transduction and selection processes are shown in the schematic FACS plot shown in the top panel of Figure 1b. The co-transduction of D-17 cells generated the following new cell populations (Figure 1b, middle panel): EGFP and mCherry double positive cells derived from the random integration events of the repair

template; EGFP and mCherry double negative cells that were predicted to correspond to targets repaired through non-homologous end joining (NHEJ); and EGFP negative mCherry positive cells that originated from targeted gene correction or from the capture of the repair template at the site of the double-strand break caused by I-SceI cleavage. Analysis of the target locus from the purified EGFP and mCherry double negative fraction showed that in 8 out of 11 sequences analyzed, the target locus had undergone insertions or deletions that impaired EGFP expression (Supplementary Figure).

To eliminate the cells that randomly integrated the repair template or that captured it at the site of the break induced by I-SceI, the cultures were subjected to iterative treatments with the synthetic dimerizer AP20187. This regimen allowed for the elimination of such cells as indicated by the depletion of EGFP⁺mCherry⁺ cells as shown in Figure 1b, bottom panel. Importantly, the selection with AP20187 led to a change in the ratio of gene corrected events to random integrants from ~1:1 to ~3:1.

To select for the cells that had undergone gene correction (EGFP^{neg}mCherry⁺), the cultures were treated with O6BG, a potent inhibitor of wild-type MGMT but not MGMTP140K, and the alkylating agent BCNU. The almost complete elimination of the EGFP⁺mCherry^{neg} and EGFP^{neg}mCherry^{neg} cells demonstrates that this treatment allowed for the efficient selection of cells that express MGMTP140K (Figure 1b, bottom panel). Significantly, these results indicate that the combination of the two selection mechanisms allows for the enrichment of the cells that had undergone targeted gene correction and the elimination of unmodified or cells with random integration events. These selection mechanisms result in a change in the original ratio of gene corrected events to random integrants from ~1:1 to up to ~14:1.

To further evaluate the efficiency of the negative selection with AP20187, the presence of the iCaspase-9 transgene in cultures subjected to gene correction and to the positive and negative selection regimens was evaluated by PCR. For this, the cells were fractionated through FACS sorting into EGFP^{neg}mCherry⁺ and EGFP⁺mCherry⁺ subsets. Next, the DNA sequence corresponding to mCherry and the 3' LTR from the provirus was amplified with primers as indicated by solid arrows in Figure 1a. If the iCaspase-9 gene is present, due to the lack of homologous recombination, an amplicon of 3178 bp is produced; conversely, if the iCaspase-9 gene is absent, because of homologous recombination, the fragment produced has only 1429 bp. The results shown in Figure 1c clearly indicate that in the EGFP^{neg} mCherry⁺ subset, which contains cells that have undergone gene correction or captured the repair DNA at the I-SceI cleavage site, the most abundant species is 1429 bp in length. In contrast, in the EGFP and mCherry double positive cell fraction, which corresponds to random integration events of the repair, the most abundant species is 3178 bp. These results demonstrate that the negative selection regimen effectively depleted the cells that did not undergo gene correction and that continued to express iCaspase-9.

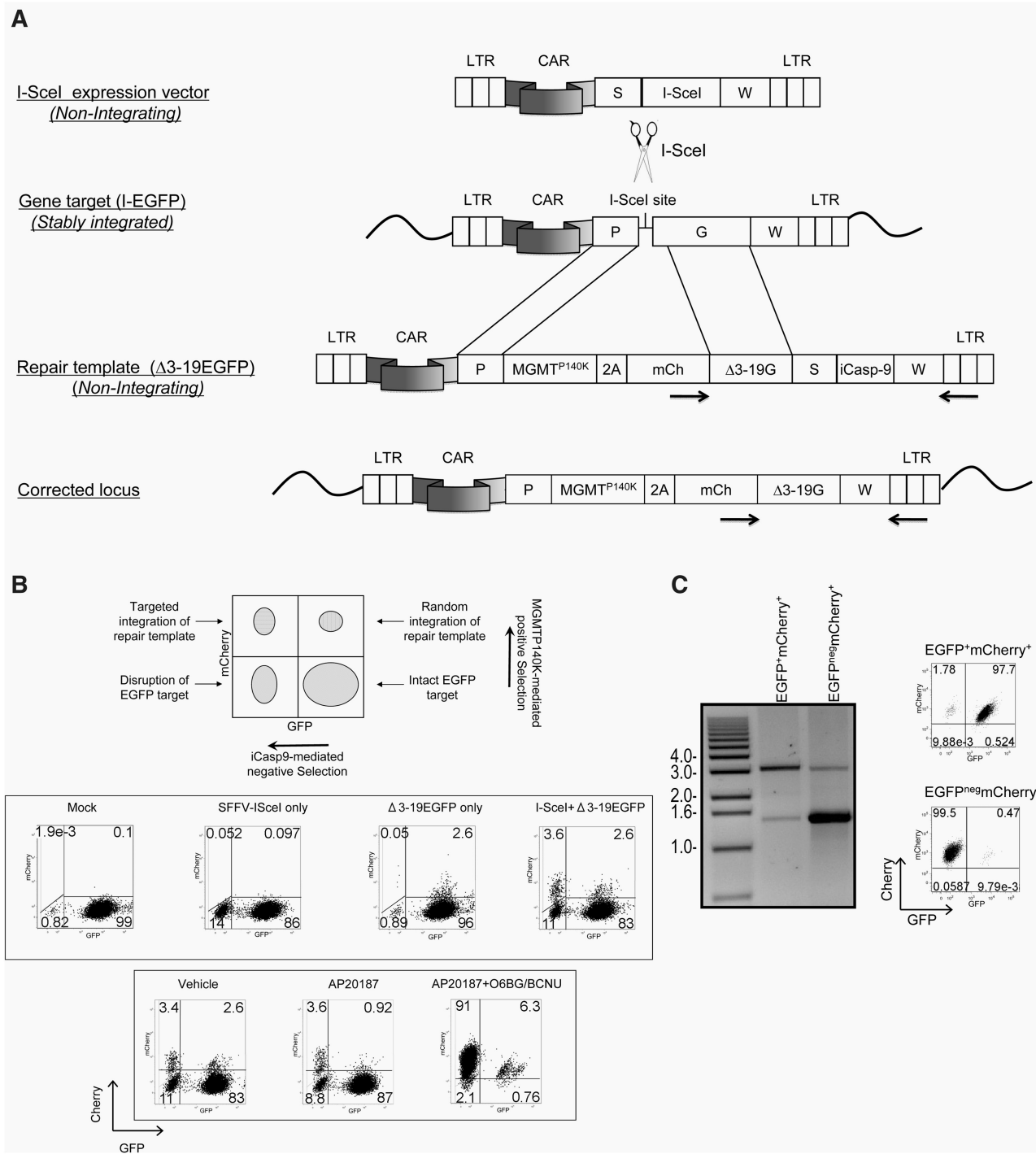


Figure 1. Gene correction on D-17 cells with the integrated I-EGFP target. **(a)** Schematic representation of the gene targeting reporter system. The non-integrating I-SceI expression vector, the integrated gene target with the I-SceI recognition site, and the non-integrating repair template are shown along with the anticipated corrected locus. Abbreviations are: LTR, long terminal repeat; CAR, *Cis*-acting region; W, Woodchuck hepatitis virus post-transcriptional regulatory element; P, Phosphoglycerate kinase promoter; G, Enhanced Green Fluorescent Protein; mCh, mCherry; S, Spleen focus-forming virus promoter. The arrows represent the primers used for detection of the iCaspase-9 gene. **(b)** Results of flow cytometry analysis. Top panel: Diagram of a FACS plot indicating the phenotypes produced after lentiviral transduction and selection of D-17 cells with the integrated target I-EGFP. Middle panel: Flow cytometry plots of cells transduced with one or two integrase-deficient lentiviral vectors that deliver the repair template or I-SceI. These are the phenotypes of the cells 45 days after transduction. Bottom panel: Treatment with the dimerizer AP20187 results in a significant reduction in the number of EGFP⁺mCherry⁺ cells, which correspond to random integrants of the repair substrate. Treatment with O6BG and BCNU to cultures already treated with AP20187 eliminates the mCherry^{neg} cells, which correspond to cells with an intact target (EGFP positive) or with a target repaired through NHEJ disrupting EGFP expression. These results are representative of two different experiments. **(c)** PCR analysis for the iCaspase-9 transgene in cells subject to gene correction and treated with AP20187 and O6BG plus BCNU. The cells were separated in EGFP⁺mCherry⁺ and EGFP^{neg}mCherry⁺ subsets through FACS sorting (FACS plots shown at the right) before DNA amplification. The molecular weight markers (kb) are shown on the left.

Gene correction on D-17 cells with the integrated N-stopEGFP target

In the reporter system described above, we used FACS sorting to enrich for the cells that contain the integrated target; however, in order to generate a system that could be more easily transferred into the *in vivo* setting, we designed another reporter system that takes advantage of the MGMTP140K selection method that allows for *in vitro* and *in vivo* selection of cells with an integrated target prior to gene repair studies. Thus, in this second reporter system, the target locus, here referred to as N-stopEGFP, consisted of a mutant EGFP gene that was rendered non-functional by mutating the third and fourth codons to stop codons, and contained the recognition site for I-SceI 11-bp upstream of the non-functional EGFP start codon. The gene target locus also contained the spleen focus-forming virus (SFFV) promoter driving expression of MGMTP140K (Figure 2a). The repair substrate, named C- Δ 14EGFP, consisted of another non-functional mutant EGFP that lacked 42 bp in the 3'-end of the gene, corresponding to 14 amino acids (Figure 2a). In this reporter system, gene correction stimulated by I-SceI cleavage leads to functional EGFP expression, which is analogous to the strategy described by Elliot *et al.* for gene correction of the neomycin resistance gene (24).

D-17 cells were transduced with a lentiviral vector encoding the target locus and polyclonal cultures were selected by treatment with O6BG and BCNU. Gene marking analysis showed high levels of integrated target with cells containing on average 0.9 proviral copies per genome. Next, the cells with the integrated N-stopEGFP gene target were transduced with IDLVs that delivered I-SceI and the C- Δ 14EGFP repair template. This co-transduction resulted in expression of a functional EGFP as a result of gene correction as shown in Figure 2b. To establish an optimal ratio of I-SceI-to-repair substrate and to achieve the highest possible level of gene correction in this cell line, the cells were treated with different amounts of each IDLV and analyzed for proper gene targeting indicated by EGFP expression. The results indicated that a 1:1 ratio (Figure 2b), and 5 μ g/ml p24 of each IDLV (Figure 2c), produced the highest level of gene correction with minimal toxicity to the cells. Importantly, the number of EGFP⁺ cells generated by gene correction was stable over time.

To confirm the repair of the gene target at the molecular level, EGFP⁺ D-17 cells produced by gene correction were enriched through FACS sorting so that >90% of the cells in the culture were EGFP positive (Figure 2d). The EGFP gene was then amplified by PCR with a primer set that amplifies the target gene but not the repair substrate. Specifically, the forward primer was located in the hPGK promoter and the reverse primer was located in the region of the EGFP sequence that the repair substrate lacks in the 3'-end. Sequence analysis of 73 clones confirmed that 30 of them (41%) correspond to the corrected gene target (Figure 2e).

DISCUSSION

Here we describe the design and implementation of two reporter systems that incorporate strategies that considerably improve gene editing approaches. In the first reporter system, I-SceI-driven correction of an EGFP target locus results in clones that are converted to EGFP negative, mCherry positive, and can be selected through expression of the MGMTP140K transgene. Unwanted random integration of the repair template results in clones that become EGFP and mCherry (MGMTP140K) double positive, but because they also express the inducible Caspase-9 gene, they can be depleted using the dimerizer AP20187. With this system, we were able to significantly shift the original ratio of gene corrected events to random integrants from ~1:1 to up to ~14:1.

Because in this first system MGMTP140K expression mediates the positive selection of cells that have undergone targeted gene correction, this system parallels conditions in which the gene-corrected cells lack a selective growth advantage and therefore a selectable marker is required to improve engraftment or increase the percentage of gene-modified cells after transplantation to therapeutic levels, like is the case of β -thalassemia (25). Moreover, the incorporation of the inducible Caspase-9 marker constitutes an additional safety measure that helps circumventing the risk of malignancy due to random integration events that could lead to proto-oncogene activation. Importantly, since the MGMTP140K and the inducible Caspase-9 transgenes have been safely and efficiently used in previous *in vivo* studies (18,20), this system could also be adaptable to animal models of targeted gene correction in hematopoietic stem cells. This is a significant advantage over previously described gene targeting methods that use neomycin resistance and *Herpesvirus* thymidine kinase for positive and negative selection, respectively (26), because such genes cannot be safely used to select cells of the hematopoietic system due to their strong immunogenicity (27).

In the second reporter system, I-SceI-driven gene correction of a non-functional EGFP gene leads to functional EGFP expression. In D-17 cells with the integrated target we were able to achieve gene correction in >8% of the cells, and this population of cells remained stable over several weeks.

In this system, selection with MGMTP140K allows for the enrichment of cells with integrated targets to nearly 100% prior to gene targeting studies. This makes it a useful model system in that it mimics a genetic disease scenario, such as X-SCID or ADA-SCID, where the entire hematopoietic system has a target for gene correction and as is especially the case with X-SCID even low levels of gene correction could lead to a therapeutic benefit. A system like this will help elucidate the necessary conditions for efficient gene correction of cells of the hematopoietic system using homing endonucleases.

In summary, here we describe the development of two reporter systems to evaluate novel and sensitive strategies to enhance gene correction driven by LAGLIDAG homing endonucleases. One of these reporter systems

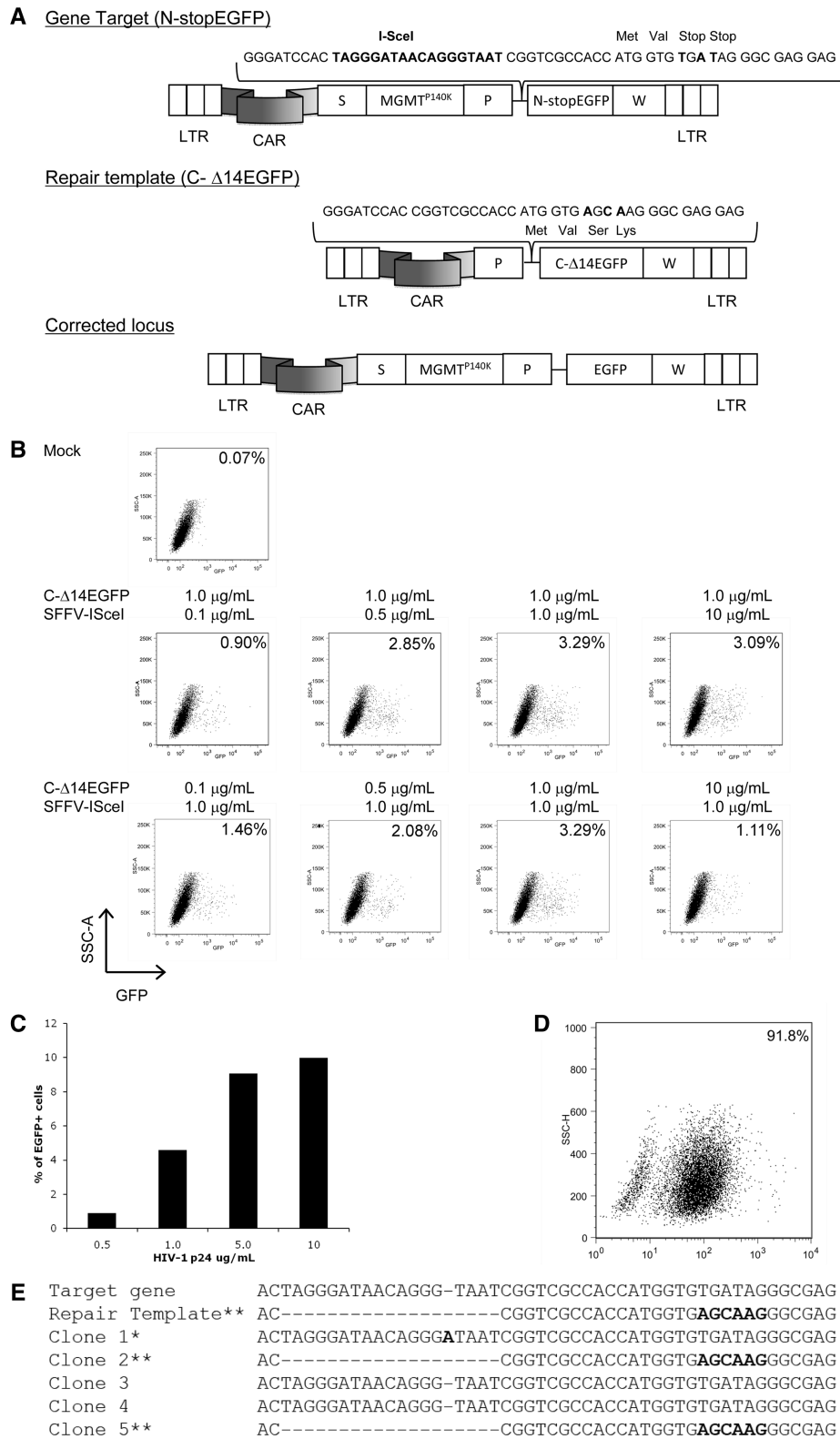


Figure 2. Gene correction on D-17 cells with the integrated N-stopEGFP target. (a) Schematic representation of the reporter system. The target locus, which was integrated into the genome using a lentiviral vector, includes a mutant EGFP gene, referred to as N-stopEGFP, with a recognition site for the I-SceI upstream of the EGFP start codon. The target also encodes the MGMT^{P140K} transgene for positive selection. The repair substrate consists of a carboxy-terminal truncated non-functional mutant EGFP, C-Δ14EGFP. The corrected locus encodes a functional copy of the EGF gene. (b) Assessment of the optimal ratio of donor DNA to I-SceI needed to achieve gene correction using IDLVs in D-17 cells. (c) Evaluation of the optimal amount of IDLVs required for maximum levels of gene correction. (d) FACS sort-mediated selection of D-17 cells with the integrated target that underwent EGFP gene correction. (e) Results of sequence analysis of the target locus in the FACS-sorted cells. The results indicate that 41% of the sequences obtained from the enriched cultures were the product of gene correction induced homologous recombination between the target and repair template (clones 2 and 5 in the subset shown here). Clone 1 demonstrated gene repair via NHEJ with 1 bp insertion in the I-SceI cleavage site.

contains the selectable markers MGMTP140K and iCaspase-9, which together allow for considerable enrichment of gene corrected cells while minimizing both random integration and NHEJ events. In the other system, selection through MGMTP140K allows for the selection of cells with the integrated gene target prior to gene correction. Thus, our approaches should facilitate safe and efficient *in vivo* applications of targeted gene correction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figure.

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