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CRISPR/Cas9-*loxP*-Mediated Gene Editing as a Novel Site-Specific Genetic Manipulation Tool

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Cre-*loxP*, as one of the site-specific genetic manipulation tools, offers a method to study the spatial and temporal regulation of gene expression/inactivation in order to decipher gene function. CRISPR/Cas9-mediated targeted genome engineering technologies are sparking a new revolution in biological research. Whether the traditional site-specific genetic manipulation tool and CRISPR/Cas9 could be combined to create a novel genetic tool for highly specific gene editing is not clear. Here, we successfully generated a CRISPR/Cas9-*loxP* system to perform gene editing in human cells, providing the proof of principle that these two technologies can be used together for the first time. We also showed that distinct non-homologous end-joining (NHEJ) patterns from CRISPR/Cas9-mediated gene editing of the targeting sequence locates at the level of plasmids (episomal) and chromosomes. Specially, the CRISPR/Cas9-mediated NHEJ pattern in the nuclear genome favors deletions (64%–68% at the human *AAVS1* locus versus 4%–28% plasmid DNA). CRISPR/Cas9-*loxP*, a novel site-specific genetic manipulation tool, offers a platform for the dissection of gene function and molecular insights into DNA-repair pathways.

INTRODUCTION

Genome editing has recently emerged as a powerful technique that allows any chosen gene to be precisely modified in a predetermined way.[1,2] The Cre-*loxP* system is a popular and widely used site-specific genetic manipulation tool to conditionally knockout specific genes in cell lines and animal models, which modulates expression of selected genes at a certain developmental stage or in specific tissues, greatly facilitating our understanding of gene function and developmental mechanisms[3–8] as well as Flp/*FRT*.[8] Cre recombinase originates from P1 phage and recognizes the 34-bp *loxP* site (5′ATAACTTCGTATAatgtatgcTATACGAAGTTAT-3′), whereas Flp recombinase originates from the yeast 2-μm plasmid and recognizes the distinct 34-bp *FRT* site (5′-GAAGTTCCTATTCtctagaaaGTATAGGAACTTC-3′).[8] Both Cre and Flp recombinase can excise a region of DNA surrounded by two *loxP* or *FRT* sites, respectively.

At the present time, the choice of tools for site-specific genetic manipulation is limited to just Cre-*loxP*, Flp-*FRT*, phiC31/*attP*, or *attB* and the Dre recombinase/*Rox*.[8–10] Additional genetic tools would be of benefit in the genetic modification of cell lines, especially to achieve genetic modification of both alleles. In vivo, double-allele knock out can be obtained by the mating of single-allele knockout animals. However, this strategy is not practical for cell lines. For this reason, new site-specific genetic manipulation tools are still highly desired.

CRISPR/Cas9-mediated targeted genome engineering technologies are sparking a new revolution in biological research.[11] CRISPR/Cas9 technology has been widely applied for functional genomic studies in a variety of organisms, including mouse and human cells.[12,13] It can be programmed by single-guide RNAs (sgRNAs) to cleave specific genomic loci complementary to the sgRNA with a downstream protospacer adjacent motif (PAM), where Cas9 creates double-stranded DNA breaks (DSBs). These DSBs mediate error-prone non-homologous end-joining (NHEJ) or precise homologous recombination (HR).[14] The most widely used customized CRISPR/Cas9 (SpCas9) is derived from *Streptococcus pyogenes*, and the corresponding PAM sequence is 5′-NGG-3′, where N is any base.[15]

Whether the traditional site-specific genetic manipulation tool and CRISPR technologies can be combined as a novel genetic tool for specific gene knockout studies is not clear (Figure S1). To address this question, we took advantage of the double fluorescent reporter systems with CRISPR/Cas9 because it is easily detectable by flow cytometry and microscopy.[16] This approach was used to test the efficiency of a CRISPR/Cas9-*loxP* system in human cells.

RESULTS

Performance of a CRISPR/SpCas9-*eloxP* System with Transient Reporter-Gene Expression

To develop a simple reporter system for visualizing CRISPR/SpCas9-*loxP* effects, we used the *mT*/*mG* system,[16] with the structure

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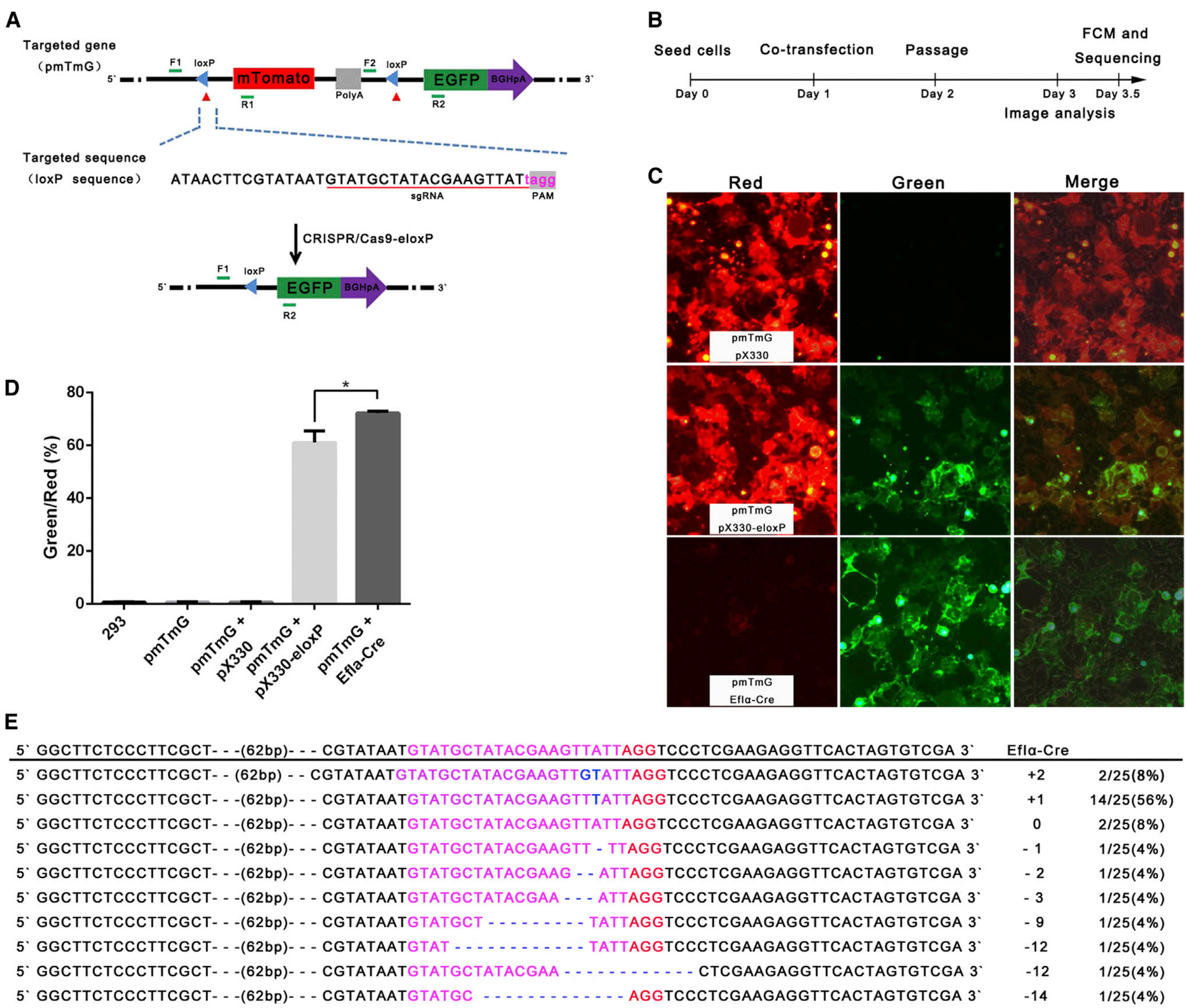

Figure 1. Performance of CRISPR/SpCas9-*eloxP* System in Transient Reporter-Gene Expression Assays

(A) Schematic of the *mT/mG* cassette (*loxP-mT*-pA-*loxP-mG*-pA) before and after CRISPR/SpCas9-*eloxP*-mediated recombination. *mT/mG* consists of a promoter driving a *loxP*-flanked coding sequence of membrane-targeted tandem dimer Tomato (*mT*), resulting in mTomato expression with membrane localization. After CRISPR/SpCas9-*eloxP*-mediated recombination, the mTomato sequence is excised, allowing the promoter to drive expression of membrane-targeted EGFP (*mG*). (B) An illustration of the CRISPR/SpCas9-*eloxP* system in transient reporter-gene expression assays. (C) HEK293 cells were co-transfected with pmTmG and CRISPR/SpCas9-*eloxP* (0.75 ug each), and images were obtained at 48 hr post transfection (red indicates *mT*; green indicates *mG*). See also Figure S2. (D) The level of fluorescence was quantified (red indicates *mT*; green indicates *mG*). Error bars are SD (n = 3). The p value was calculated by Student's t test; *p < 0.05. (E) NHEJ pattern of CRISPR/SpCas9-*eloxP*-mediated gene editing. Pink nucleotides indicate sgRNA. Blue nucleotides indicate indels.

CAG(promoter)-*loxP*-mTomato(mT)-*loxP*-Poly(A)- EGFP (mGreen, mG)-Poly(A) (Figure 1A). We hypothesized that if we introduced two CRISPR/Cas9-mediated DNA double strands break via targeting *loxP* flanking the *mTomato* cassette, the expression of the *EGFP* gene will be directly driven by the CAG promoter. Because there are two identical *loxP* sequences flanking the *mTomato* cassette, one sgRNA would be able to target them. However, *loxP* sites do not contain an "NGG" PAM sequence, and the additional tAGG sequence at the 3′ end of the *loxP* (5′-ATAACTTCGTATAAtgtatgcTATACGAAGTTATtagg-3′), named extended *loxP* (e*loxP*), was created (Figure 1A; Table S1). Thus, the reformed *loxP* with the addition of an NGG PAM sequence can be recognized by CRISPR /SpCas9. As we expected, co-transfection of two plasmids (pX330- *eloxP* expressing Cas9 and sgRNAs to target *eloxP* and pmTmG) in human HEK293 cells indeed led to EGFP expression (Figure 1C; the schematic of the protocol is shown in Figure 1B), which implied that the CRISPR/SpCas9-*eloxP* system can be used to excise the mTomato gene and allow the CAG promoter to drive the expression of the EGFP gene directly. Specially, in the CRISPR/SpCas9-*eloxP* group, 56.0%–63.6% of cells were EGFP-positive cells (Figures 1C, 1D, and S2). PCR was then used to confirm the NHEJ events triggered appropriately by CRISPR/SpCas9-*eloxP* (Figure 1E).

To gain insights into the occurrence of the NHEJ events, we mapped the sequences of CRISPR/Cas9-*eloxP*-mediated NHEJ products. Comparing one *loxP* sequence left in the Cre group, notably, there was one dominant sequence (named −3T Ins, 14/25, 56%) in the CRISPR/Cas9-*eloxP* group, which comprises the insertion of one additional T base 3 nt upstream of the PAM (Figure 1E). Other fusion sites included one *loxP* sequence, an additional GT at 3 nt upstream of the PAM as well as 1-, 2-, 3-, 9-, 12-, and 14-bp multiple variable deletions at lower frequencies (Figure 1E). The majority (64%) of the fusion sites have insertions. Taken together, with plasmids (transient reporter-gene expression), our data showed that CRISPR/Cas9-*eloxP* system-based gene editing can achieve high excision efficiency of a specific gene between two *loxP* sites.

Generation of a Double-Fluorescent Reporter System at the Human *AAVS1* Locus

Because our system showed high excision efficiency on the specific gene between two *loxP* sites at the plasmid co-transfection level, we wondered whether it would also be functional at human chromosomal loci. To address this, we sought to generate a double-fluorescent reporter system at the human *AAVS1* locus. *AAVS1* (also known as the *PPP1R12C* locus) on human chromosome 19 is a well-validated "safe harbor" for hosting DNA transgenes.[17–19] It has an open chromatin structure and contains native insulators that prevent the integrated genes silencing.[17] Most importantly, there are no known adverse effects on cells as a result of DNA fragment insertion. For the above reasons, we selected *AAVS1* as a targeting site for harboring the double-fluorescent reporter system.

We designed one sgRNA targeting the *AAVS1* locus to introduce DSBs and the pmTmG plasmid, which carries two arms homologous to the *AAVS1* locus, as the donor vector to knock in a double-fluorescent reporter gene at the human *AAVS1* locus (Figures 2A and 2B). To screen positive colonies, which maintain robust mTomato gene expression under the microscope (Figure 2C), we initially picked colonies that showed red fluorescence under the microscope. Then, we re-picked the colonies among the red fluorescent candidates and assumed that most random integration events will lose the red fluorescent signal, whereas the knockin cell line would maintain red fluorescence. After six passages of the re-picked candidate colonies, we screened them with gene-specific PCR (Figure 2D; Table S2). Because one primer anneals to sequences located outside of the homologous arm, all the positive colonies should contain the homologous recombinant knockin gene. 8 of the 12 colonies selected were positive ones. The four PCR-negative colonies should be due to random integration of the cassette, even if it has mTomato gene expression, which would not be suitable for further experiments.

For further studies, one of the positive colonies was named 293-AAVS-mTmG, which has red fluorescence and also has a clean homogeneous genetic background. 293-AAVS-mTmG should only be a monoallelic, but not biallelic, knockin cell line because we could amplify the fragment of the wild-type *AAVS1* allele with the expected size, which indicates there is one intact wild-type *AAVS1* allele (Figure S3).

CRISPR/SpCas9-*elox*P-Mediated Site-Specific Genome Editing in 293-AAVS-mTmG Cells

The plasmids for the CRISPR/SpCas9 expression system targeting *eloxP* were transfected into 293-AAVS-mTmG, and the level of effective genome editing was measured by identifying EGFP-positive cells with flow cytometry (Figure 3A). We observed a dose-dependent effect of plasmid transfection. Specifically, transfection of 0.5, 1.5, and 2.5 μg of plasmid per well of a six-well plate achieved approximately 15.1%, 20.4%, and 24.5% recombination efficiency (Figures 3B, 3C, and S4), respectively. DNA sequence chromatograms confirmed the occurrence of NHEJ in these cells (Figure 3D). These results showed that CRISPR/SpCas9-*eloxP*-mediated site-specific genome editing has been achieved at the human *AAVS1* locus.

We then sought to ask whether there was the same NHEJ pattern between the plasmid-based NHEJs and reporter genes at the human *AAVS1* locus. After transfection and isolation of the corresponding genomic DNA from 293-AAVS-mTmG cells, fragments harboring NHEJ sites were amplified and sequenced. Surprisingly, at the human *AAVS1* locus, we did not identify the fusion sequence (one intact *loxp* sequence), which was present at the group of plasmid-based NHEJs (Figures 3E and S5). The recurrent dominant sequence (−3T Ins) was with the insertion of an additional T base 3 nt upstream of the PAM and was detected at high frequency (8/25, 32%). Also, 107-, 3-, and 2-bp deletions were the major NHEJs in addition to −3T Ins (Figure 3E). Notably, the majority (64%) of the NHEJs have deletion at the human *AAVS1* locus, whereas the majority (64%) of plasmid (transient gene expression)-based NHEJs have insertion. These results implied that the target sequence under a different environment (plasmid and at the chromosome) may affect the formation of the NHEJ pattern of CRISPR/SpCas9.

Our previous study clearly demonstrated that NAG may not be the universally predominant non-canonical PAM for CRISPR/Cas9-mediated DNA cleavage in human cells.[20]. We also tested NGA or NAG PAM, and the results showed that CRISPR/Cas9-loxP with NGA non-canonical PAM, but not NAG, could achieve relative highly efficient (10%) excision of a specific gene between two *loxP* sites (Figure S6).

CRISPR/SaCas9-eloxP-Mediated Site-Specific Genome Editing in 293-AAVS-mTmG Cells

Recently, a novel type of Cas9 (SaCas9) was derived from *Staphylococcus aureus*, and it can edit the genome with efficiencies similar to those of SpCas9, despite being 1 kb shorter.[21] Its smaller size allows packaging into a single AAV vector, with its sgRNA expression

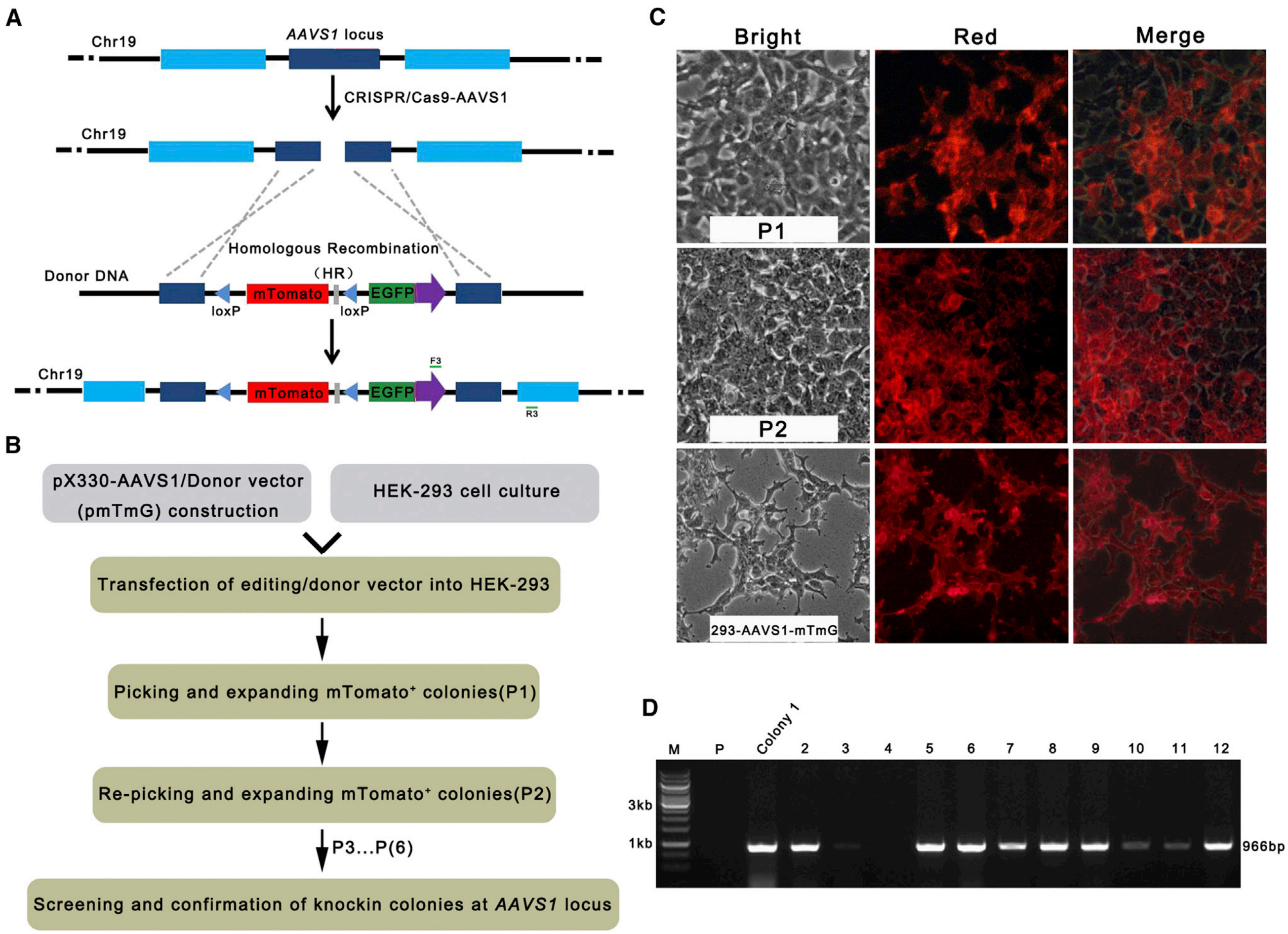

Figure 2. Generation of a Double-Fluorescent Reporter System at the Human *AAVS1* Locus

(A) Human *AAVS1* locus knockin via CRISPR/Cas9. (B) Flowchart outlining generation of gene knockin cells. (C) Images of the cells for picking up positive clones that specifically integrated *mT/mG* cassette into the *AAVS1* locus at chromosome 19. From the top to the bottom, it shows the stable knockin colonies were generated. P1, passage 1; P2, passage 2. (D) Genotyping of the *mT/mG* cassette knockin candidate colonies with F3/R3 primers.

cassette, for in vivo genome editing. The canonical PAM sequence in SaCas9 is "NNGRR(T)," and the corresponding eloxP sequence is ATAACTTCGTATAatgtatgcTATACGAAGTTAT attAAGGGT (Figure 4A).

Because site-specific genome editing is very important in vivo, here, we sought to test the performance of the CRISPR/SaCas9-*eloxP*-mediated novel site-specific genome editing in 293-AAVS-mTmG cells. We observed a relatively high efficiency of CRISPR/SaCas9-*eloxP*-mediated genome editing (27.6%–31.4% versus 19.5%–24.9%; Figures 4B and S7). Also, we found co-transfection of CRISPR/Cas9- and pmTmG-based gene editing could achieve a higher efficiency comparing genome editing at the *AAVS1* locus (Figure 4B). Like the CRISPR/SpCas9-*eloxP* at *AAVS1* locus, the majority (64%) of the fusion site (NHEJs) has deletion, including 2- to 37-bp deletion (Figures 4C and S8). The results of the NHEJ patterns of transient CRISPR/SaCas9-*eloxP* expression showed that the minority (8%) of the fusion site is deletion, which is different from that of the CRISPR/SaCas9-*eloxP* at *AAVS1* locus (64% of the fusion sites have deletions).

Taken together, our study demonstrated that CRISPR/Cas9-*loxP* (Sa Cas9 or Sp Cas9) could be used as a tool to perform site-specific genome editing at the human *AAVS1* locus. Meanwhile, CRISPR/Cas9-mediated NHEJ patterns may be modulated by the environment of targeting sequence.

Off-Target of CRISPR/SaCas9-*loxP*-Mediated Site-Specific Genome Editing

Off-target effects of CRISPR/Cas9 remain a key issue for its application in genome editing, including PAM-related off-target effects.[20,22] In cell lines, it is not practical to re-correct the off-target effects, although these could be minimized through outcrossing in animals or plants. New strategies using double nickase (DN) and

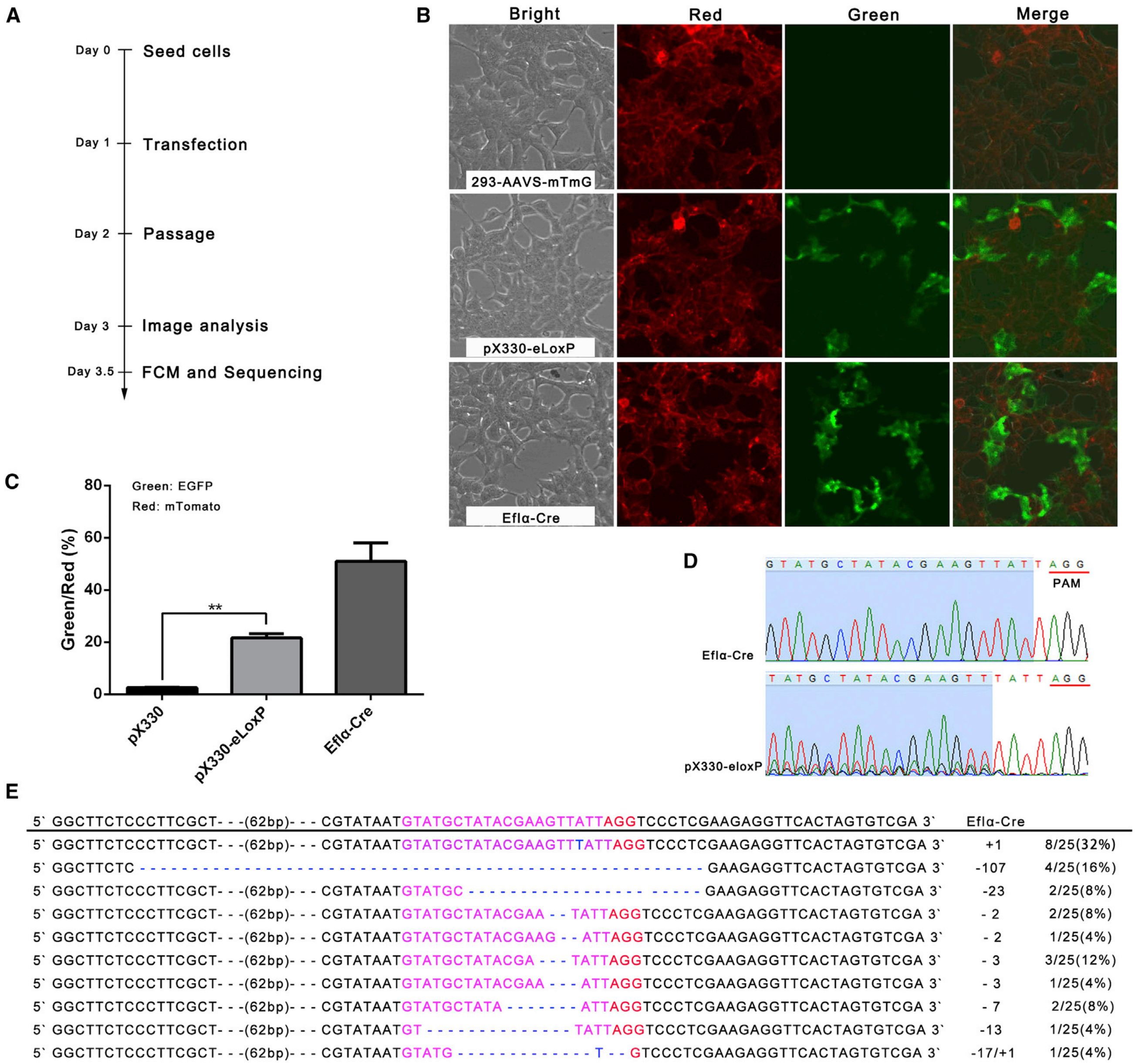

Figure 3. CRISPR/SpCas9-*eloxP*-Mediated Site-Specific Gene Editing in 293-AAVS-mTmG Cells

(A) An illustration of the CRISPR/SpCas9-*eloxP* system that mediated site-specific gene editing in 293-AAVS-mTmG cells. (B) 293-AAVS-mTmG cells were transfected with 2.0 ug plasmids (plasmids coding for Cre or CRISPR/SpCas9-*eloxP*), and images were obtained at 48 hr post-transfection (red indicates *mT*; green indicates *mG*). (C) The level of green fluorescence was quantified. Error bars are SD (n = 3). The p value was calculated by Student's t test; **p < 0.01. (D) Targeting DNA sequence chromatograms of cells transfected with CRISPR/SpCas9-*eloxP* are clustered together compared with one *loxP* remaining in the Cre group. (E) CRISPR/SpCas9-*eloxP*-mediated NHEJ pattern at the human *AAVS1* locus. Pink nucleotides indicate sgRNA. Blue nucleotides indicate indels.

FokI-dCas9 have been proposed,[23,24] but the presence of off-target effects due to Cas9/sgRNA may still exist. In the present study, we sought to know the off-targets of CRISPR/Cas9-*eloxP*. With online software (http://www.rgenome.net/cas-offinder/), we predicted the potential off-target sites in the human genome. 20 fragments harboring potential off-target sites for CRISPR/SaCas9-*eloxP* and CRISPR/SpCas9-*eloxP* have been amplified and sequenced (Tables S3 and S4). Compared with the parental cells, no additional multi-peaks in the chromatogram have been observed around the potential off-target sites (Table S4). This indicated that there are non-detectable off-target effects of CRISPR/Cas9-*eloxP* by Sanger sequencing. Further studies also need to characterize additional potential genome modifications inducing off-target CRISPR/Cas9-*eloxP* with a genome analysis tool, i.e., whole genome sequencing. Meanwhile, it is

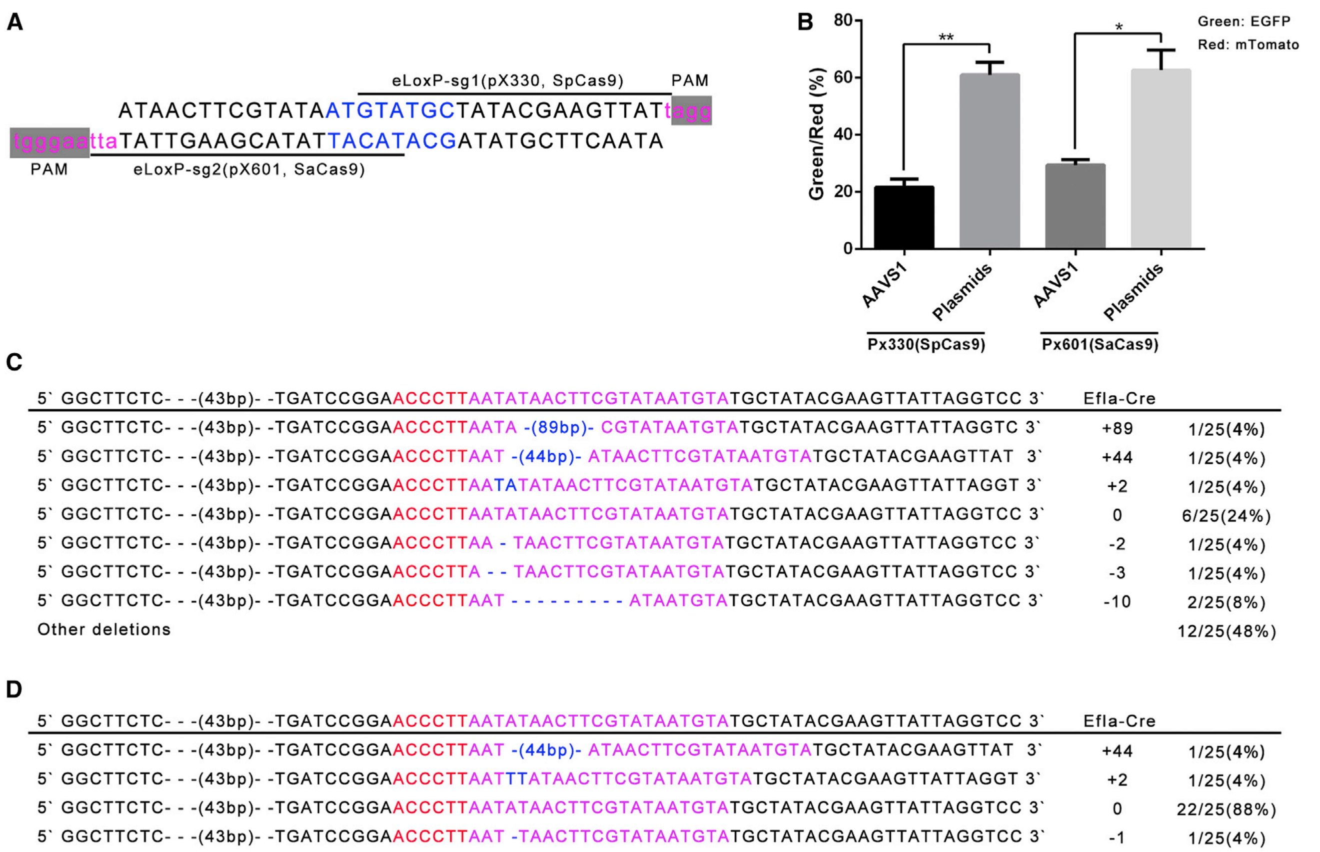

Figure 4. CRISPR/SaCas9-*eloxP*-Mediated Site-Specific Gene Editing in 293-AAVS-mTmG Cells

(A) Targeting sequence and corresponding PAMs for CRISPR/SpCas9 (pX330) and CRISPR/SaCas9 (pX601). (B) Efficiency comparison of CRISPR/SaCas9 and SpCas9-*eloxP* mediated gene editing. The p value was calculated by Student's t test; $^{*}p < 0.05$; $^{**}p < 0.01$. (C) CRISPR/SaCas9-*eloxP*-mediated NHEJ pattern at the human *AAVS1* locus. Pink nucleotides indicate sgRNA. Blue nucleotides indicate indels. See also Figure S5. (D) CRISPR/SaCas9-*eloxP*-mediated NHEJ pattern in transient reporter-gene expression assays. Pink nucleotides indicate sgRNA. Blue nucleotides indicate indels.

meaningful to screen and identify specific Cas9 mutants, which are optimized for genome engineering to minimize the off-target effects.[25] Recently, novel high-fidelity Cas9s have been reported, which may strengthen the present novel CRISPR/Cas9-*eloxP* tool.[26,27]

DISCUSSION

In the present study, we successfully generated a CRISPR/Cas9-*loxP* system to perform site-specific genome editing in human cells, at the level of both plasmids (episomal) and chromosomes. It provides the proof of principle that these two technologies (traditional site-specific genetic manipulation tool and CRISPR technologies) can be used together. We also showed that distinct NHEJ patterns from CRISPR/Cas9-mediated gene editing of the targeting sequence locates at the level of plasmids (episomal) and chromosomes.

Although CRISPR/Cas9-*eloxP*-based knockout results are certainly encouraging, improvements still need be made. For example, if we place a specifically mutated *loxP* flanking the targeting gene, which Cre cannot recognize, with the specific sgRNA-targeting mutated *loxP*, it could be used to perform conditional knockouts, and thus could be an attractive alternative genetic tool for the compensation of Cre-*loxP*. The same strategy may be applied for the Flp-FRT or even two identical sequences at different locations in the genome. In the present study, we used *eloxP* for CRISPR/Cas9. There are also two *loxP* mutants, loxP66 and loxP71, which both have NGG sequences or NNGRR(T) (Figure 5A).[28] Thus, these two additional *loxP* mutants may be more suitable for CRISPR/Cas9-*loxP*-mediated genome editing. Also, the wild-type and *loxP* mutants could be combined and the specific gene flanking them could be excised. We propose that additional CRISPR/SaCas9-*loxP* genetic tools could be developed (Figure 5B). For example, as illustrated in Figure 5B, genes A–D were flanked by different *loxP* sites. With Cre-mediated gene recombination, genes A and C could be excised. Additional sgRNAs targeting *loxP* or its mutants could allow different genes to be selectively excised or activated. Meanwhile, tissue-specific or inducible promoter could be used to drive Cas9 expression for conditional gene knockouts for animal models and in human embryonic stem cell or induced pluripotent stem cell studies.[29,30] These tools will be useful for conditional knockout and studies of gene-gene interaction.

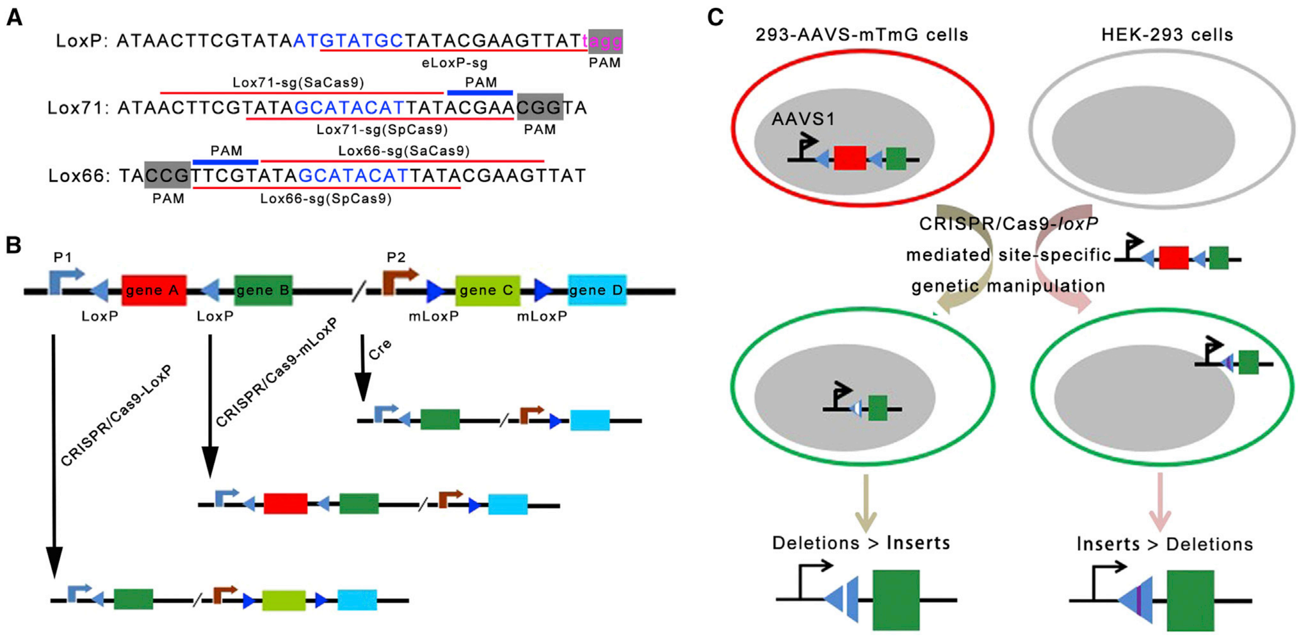

Figure 5. CRISPR/Cas9-*loxP*-Mediated Gene Editing as a Novel Site-Specific Genetic Manipulation Tool
(A) Schematics illustrating the wild *loxP* and mutant *loxP* pair lox66/lox71 sequence and the position of the guide RNA. (B) Schematic strategy of the switch gene expression employing the CRISPR/Cas9-*loxP* system. (C) Distinct NHEJ patterns from CRISPR/Cas9-mediated gene editing of the targeting sequence located at the level of plasmids (episomal) and chromosomes.

We also showed that there is a difference between the CRISPR/Cas9-mediated NHEJ patterns of the targeting sequence located at the level of plasmids (episomal) and chromosomes. In particular, the CRISPR/Cas9-mediated NHEJ pattern in the nuclear genome is in favor of deletions (64%–68% at the human *AAVS1* locus versus 8%–28% plasmid DNA). We speculate that the difference between these two groups may be due to one of the following mechanisms (Figure 5C). First, the nuclear genome and plasmid DNA exist in different genetic environments, with the nuclear genome heavily regulated by histones and other DNA-interacting proteins,[31] whereas the non-nuclear genome, including plasmids as episomes, are naked, with very few proteins binding. Second, different NHEJ-related enzymes may be involved or they may be at different concentrations in the two scenarios. This study may provide insights for NHEJ-related DNA repair and provides a platform for the comparison of chromosomal and extra-chromosomal DNA repair.

It is challenging but of interest to compare CRISPR/Cas9 editing of the same DNA sequence integrated into the nuclear genome (at the chromosomal level) and extra-chromosomal DNA (such as plasmids transiently existing in the cells). Here, we demonstrated that with the same DNA target sequence and CRISPR/Cas9, in the same cell type, extra-chromosomal genome CRISPR/Cas9-based gene editing could achieve a high efficiency compared with gene editing at chromosomes (55.0%–69.0% versus 19.5%–31.4% at the *AAVS1* locus). We rationalize the results from these two groups are not readily comparable because there are still several factors that affect the comparison, especially target sequence copy number, which is higher in the case of plasmid transfection. This still has important implications for the future extensive use of this genome engineering to edit additional DNA outside the nuclear genome, such as in virus-related disease[32] and mitochondrial diseases.[33]

In summary, we demonstrated that CRISPR/Cas9-*loxP*, a novel site-specific genetic manipulation tool, offers a genetic platform for the dissection of gene function and molecular insights into DNA-repair pathways.

MATERIALS AND METHODS

Plasmid Information

The vector plasmid pmTmG contains the following: mTomato, EGFP, two *loxP* sites, and two homologous arms (Figures 1A and S1). It is originally from Dr. Murry Charles (University of Washington). The Cre-expression vector was generated using the *Cre* gene downstream of the elongation factor-1 alpha (EFIα) promoter. Plasmids pX330 and pX601 were gifts from Feng Zhang (Addgene plasmid # 42230 and # 61591). sgRNA oligos were annealed and cloned into the pX330 or pX601 vectors using a standard protocol. Plasmid DNA was isolated by standard techniques. DNA sequencing confirmed the desired specific sequences in the constructs.

Cells and Cell Culture

HEK293 cells were cultured as previously described.[21] To generate *AAVS1* knockin cell lines containing *mT*/*mG* expression cassettes, HEK293 cells were seeded on day 0 at 2.5 × 10^5 cells in six-well plates, and on day 1, pmTmG and pX330-*AAVS1* plasmids were transfected by the calcium-phosphate precipitation method. Individual clonony with the expression of red fluorescent reporter genes were picked under the microscope at day 15. PCR was used for the confirmation of the gene knockin at the *AAVS1* locus with specific primers, which is shown in Figure 2. The cell line with *mT/mG* expression cassette knockin at the *AAVS1* locus was named 293-AAVS-mTmG.

Images and Flow Cytometry Analysis

On day 0, 5 × 10^5 293-AAVS-mTmG cells were seeded in six-well plates. On day 1, the cells were transfected with pX330-*eLoxP* or EfIα-Cre plasmids by the calcium-phosphate precipitation method. On day 2, the transfected 293-AAVS-mTmG cells were treated with trypsin and replated in a six-well plate. On day 3, the expression of red/green fluorescent reporter genes was observed under the microscope. On day 3.5, cells were harvested for flow cytometry and genomic DNA isolation (Figure 2A). Quantification was based on relative fluorescent frequencies. Green/red percentage was determined by the formula $100 \times (a/(1-b))$, where a is the green fluorescent frequencies and b is the no fluorescent frequencies.

Purification of Genomic DNA and Sequencing

Genomic DNA was purified from cells using the standard phenol/chloroform extraction protocols. The reporter gene sequence flanking the CRISPR target site was PCR amplified, and products were cloned into the vector pJET1.2 (CloneJET PCR Cloning Kit, Thermo Fisher Scientific). The PCR products and vectors were purified and then sequenced on an ABI PRISM 3730 DNA Sequencer (sequencing primers are shown in Table S1).

Off-Target Analysis for CRISPR/SpCas9-*eloxP*

We examined the possibility that CRISPR/Cas9-*eloxP* induced off-target mutations in 293-AAVS-mTmG. The potential off-target sites were predicted using online software (http://www.rgenome.net/cas-offinder/). The fragments harboring potential off-target sites have been amplified (primer information in Tables S3 and S4) and sequenced on an ABI PRISM 3730 DNA Sequencer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.018.

AUTHOR CONTRIBUTIONS

F.G. conceived the idea; F.Y., C.L., D.C., M.T., H.X., H.S., X.G., L.T., and J.Z. performed the experiments; J.L., Z.S., J.Q., and F.G. performed data analyses; and F.G. wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that they have no competing interests.

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Supplemental Information

CRISPR/Cas9-*loxP*-Mediated Gene Editing as a Novel Site-Specific Genetic Manipulation Tool

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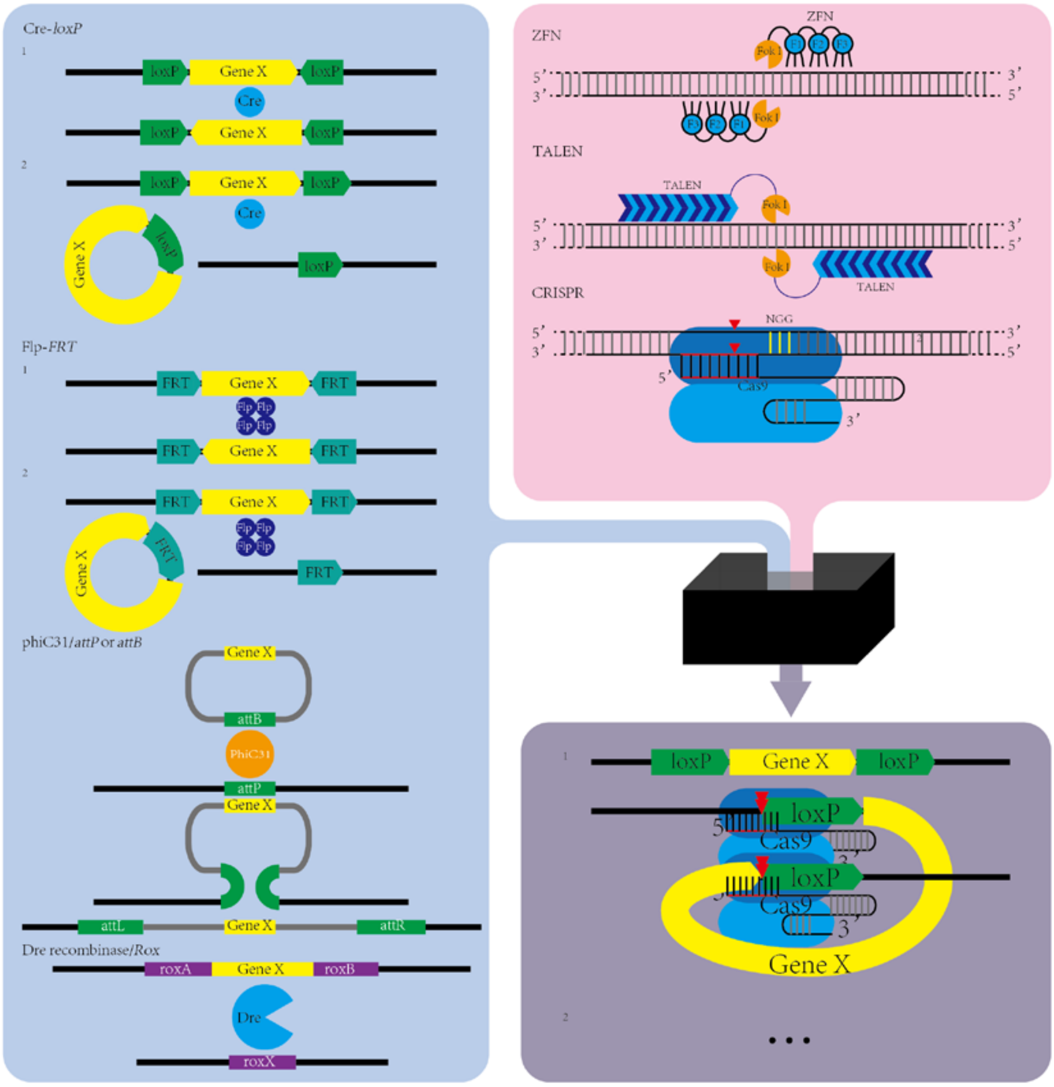

Figure S1. Schematic of the combination of CRISPR/Cas9 and traditional site-specific genetic manipulation tools.

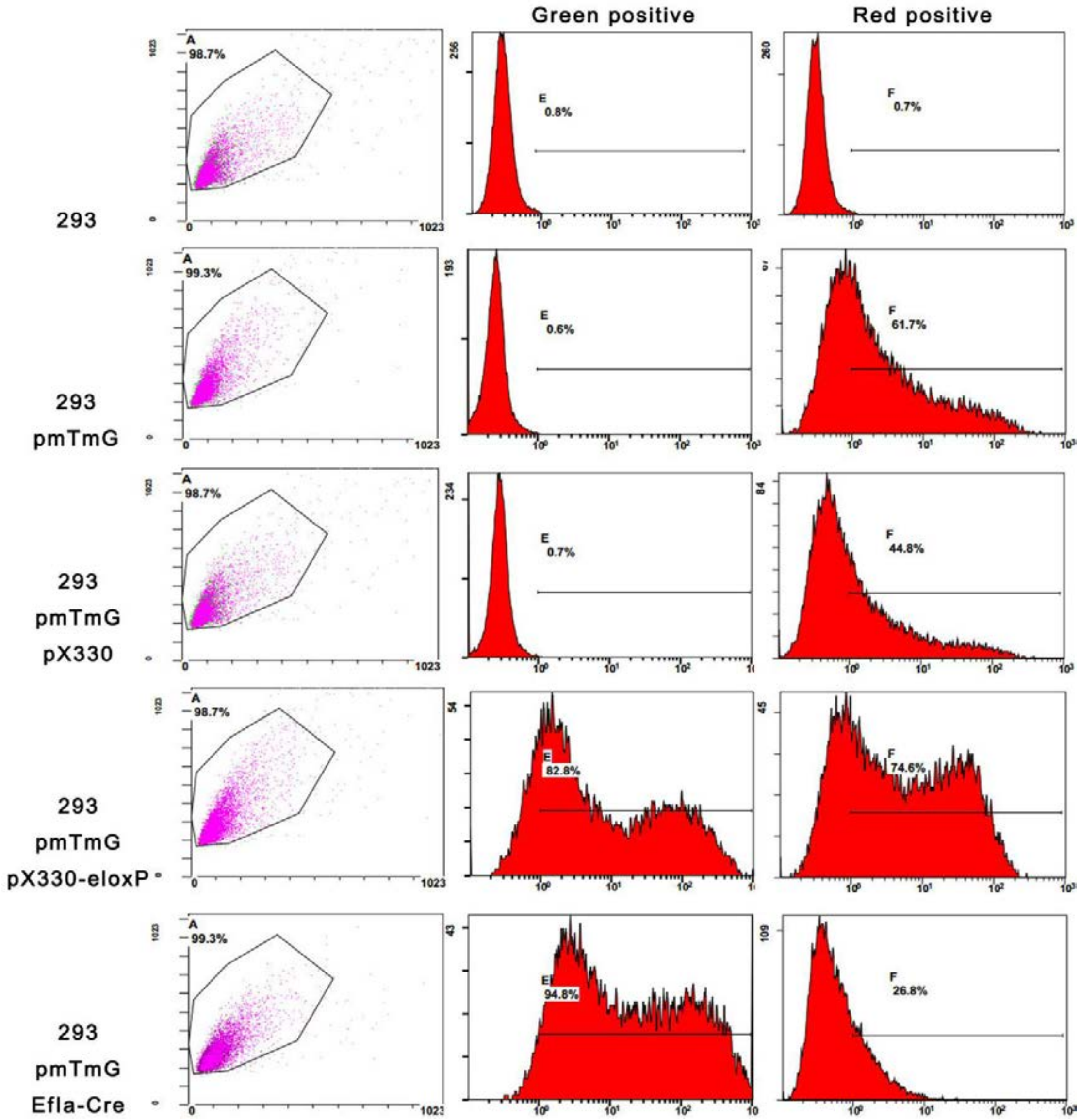

Figure S2. Performance of CRISPR/SpCas9-*eloxP* mediated site-specific genome editing examined with flow cytometry (FCM)

HEK-293 cells were co-transfected with plasmids (pmTmG and CRISPR/SpCas9-*eloxP*,0.75ug each) and flow cytometry were performed at 60h post transfection (Red indicates *mT;* Green indicates *mG*).

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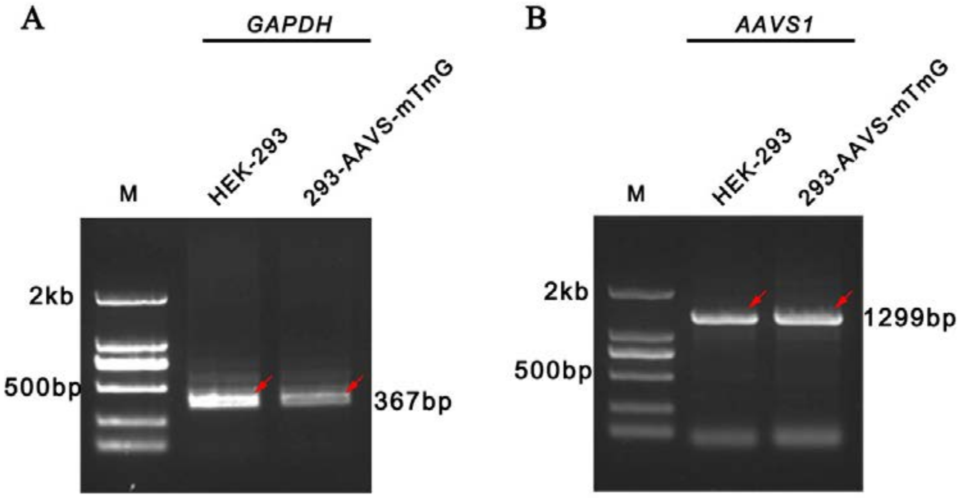

Figure S3. Genotyping of 293-AAVS-mTmG at *AAVS1* locus.

A. PCR products of *GAPDH* gene as control (Primers: F4/R4).

B. Genotyping of the *AAVS1* gene in 293-AAVS-mTmG and parental cells (Primers: F5/R5). The unlabelled bands are the primer dimer.

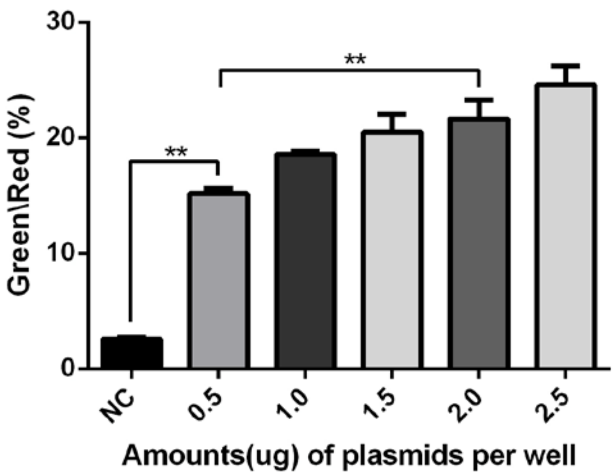

Figure S4. Optimization of transfection conditions of CRISPR/SpCas9 -*eloxP* plasmid to inactivate *mT*.

293-AAVS-mTmG cells were transfected with different amounts of CRISPR/SpCas9-*eloxP* plasmid. The cells were saturated with 2.0 mg of CRISPR/SpCas9-*eloxP* plasmids.

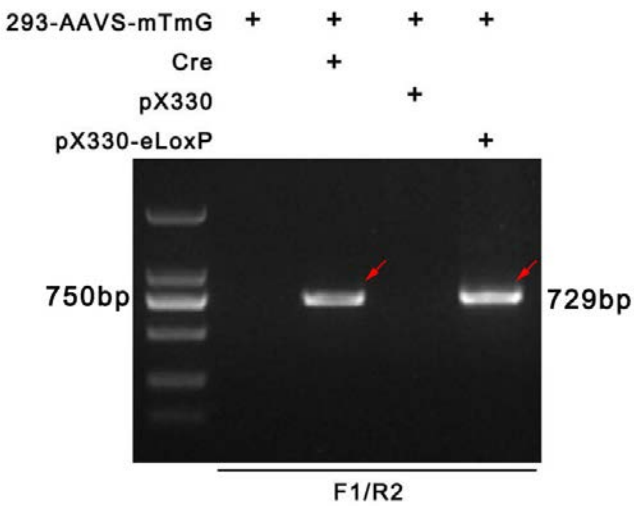

Figure S5. Genotyping of CRISPR/SpCas9-*eloxP* system mediated excision at human *AAVS1* locus.

CRISPR/SpCas9-*eloxP* mediated excision of mTomoto between two *loxP* sites in 293-AAVS-mTmG. Excision bands were detected by PCR (Primers: F1/R2).

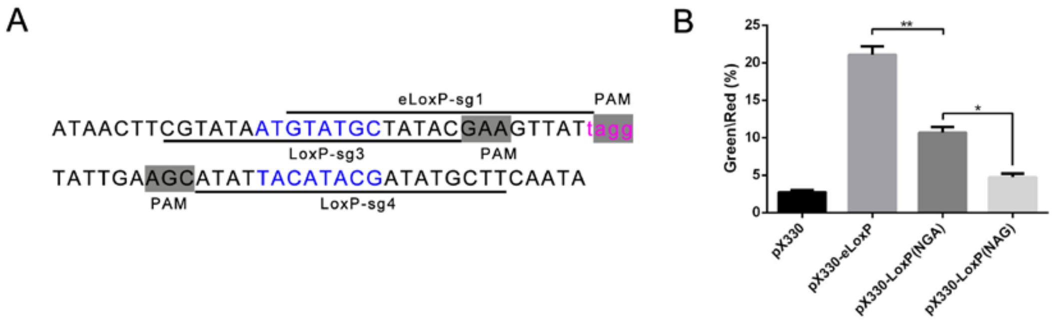

Figure S6. Non-canonical PAM for CRISPR/SpCas9-*loxP* to inactivate *mT*.

A. Targeting sequence and corresponding PAMs for CRISPR /SpCas9.

B. NGA or NAG PAM and the results showed that CRISPR/SpCas9-*loxP* with non-canonical PAM NGA but not NAG could achieve relative highly efficient excision of a specific gene between two *loxP* sites.

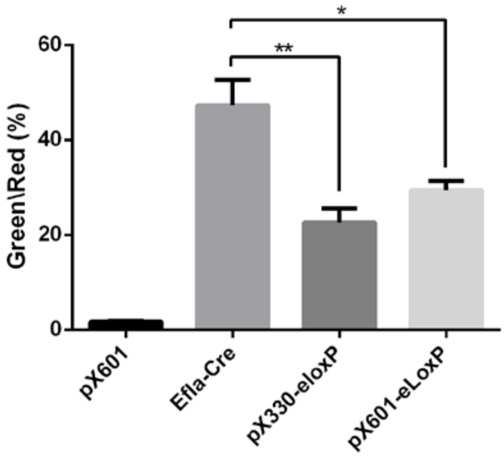

Figure S7. Efficiency comparison of CRISPR/SaCas9-*eloxP* and SpCas9-*eloxP* mediated gene editing.

pX330 and pX601 represent CRISPR /SpCas9 and CRISPR/SaCas9, respectively. Error bars are the standard deviation (SD, n=3).

Figure S8. CRISPR/ SaCas9-*eloxP* mediated NHEJ pattern at the human *AAVS1* locus.

CRISPR/SpCas9-*eloxP* mediated excision of mTomoto between two *loxP* sites in 293-AAVS-mTmG. NHEJ pattern were detected by sequencing. Pink nucleotides indicate sgRNA, blue nucleotides indicate indels.

Table S1 Target sequence

Target sites for CRISPR/Cas9 System with PAMs

Target site ID	Target sequence (5`-3`)	PAM	Strand
eLoxP-1(pX330)	GTATGCTATACGAAGTTATT	AGG	+
eLoxP-2(pX601)	TACATTATACGAAGTTATATT	AAGGGT	-
AAVS1	TCACCAATCCTGTCCCTAG	TGG	+
LoxP-3(pX330)	CGTATAGCATACATTATACG	NAG	+
LoxP-4(pX330)	TTCGTATAATGTATGCTATA	NGA	-

Oligonucleotide sequences for sgRNA architecture

Primer Name	Primer sequence (5`-3`)
eLoxP-1-F	CACCGGTATGCTATACGAAGTTATT
eLoxP-1-R	AAACAATAACTTCGTATAGCATACC
eLoxP-2-F	CACCGTACATTATACGAAGTTATATT
eLoxP-2-R	AAACAATATAACTTCGTATAATGTAC
AAVS1-F	CACCGTCACCAATCCTGTCCCTAG
AAVS1-R	AAACCTAGGGACAGGATTGGTGAC
LoxP-3-F	CACCGCGTATAGCATACATTATACG
LoxP-3-R	AAACCGTATAATGTATGCTATACGC
LoxP-4-F	CACCGTTCGTATAATGTATGCTATA
LoxP-4-R	AAACTATAGCATACATTATACGAAC

Table S2 Primers sequences

PCR primers

Primer Name	Primer sequence (5`-3`)	Product(bp)	Anneaing(℃)
F1	GGGACTTCCTTTGTCCCAAATC	740bp	58
R1	GGGAAGGACAGCTTCTTGTAATC		
F2	CAGGCATAGAGTGTCTGCTATT	725bp	58
R2	GATGAACTTCAGGGTCAGCTT		
F3	GGGCTATGAACTAATGACGGA	966bp	60
R3	GTCCAGGCCAAGTAGGTG		
F4	CTGGCACCCTATGGACACG	367bp	60
R4	GTCTTCTGGGTGGCAGTGAT		
F5	ACCTCTCACTCCTTTCATTTGG	1299bp	60
R5	TGAGTTTGCCAAGCAGTCA		
F6	CTCTCGTTTCTTAGGATGGCC	150	60
R6	GAAAGCAAGAGGATGGAGAGG		

Sequencing primers

Primer Name	Primer sequence (5`-3`)
U6-F	GAGGGCCTATTTCCCATGATTC
Jet-F	TGAACACCATATCCATCCGGCGTA

Table S3 Off-target analysis for CRISPR/SpCas9-*loxP* System

PCR primers

Primer Name	Primer sequence (5`-3`)	Product(bp)	Anneaing(℃)
F7	GACAGAGAGGCACAAAGTCATA	305bp	58
R7	AGAATACTGGTCCCTGGAAATG		
F8	GAGACGGAGTCTTGTTCCATAG	499bp	58
R8	GTGTTGTTAGTTGGAAGAGTCATTAG		
F9	GCCATGATGACTTCACCTGAT	455bp	58
R9	TTGGTTTGGGCAATACCTTCT		
F10	AGCTGACTTCTCACCACAAAC	374bp	58
R10	GCAATGACACAATTGCCTAACC		
F11	TGGAGATTAAGAGACAAGGGAATG	458bp	58
R11	GTAATATCACAGCTACTTGGAGAGG		
F12	GGCAGAGAAGGGCAAGTAAG	348bp	58
R12	GCCACGTCAGTAGTGGTATTT		
F13	GCATCAGAAAGCACAGAGACTA	441bp	58
R13	GCTTCCTTTACTCTCACCCTTTA		
F14	ACAGTGGTAAAGACCAATCAGG	300bp	58
R14	CTGCTGTGTACCTATGTCAGAAG		
F15	TGGAGATTAAGAGACAAGGGAATG	458bp	58
R15	GTAATATCACAGCTACTTGGAGAGG		
F16	TGGTGCGTGCCTGTAATC	420bp	58
F16	CCAGTTGCCTTTGAAGTCATTC		

Analysis of off-target induced by CRISPR/SpCas9-*loxP* System

Chromosome	Target sequence (5`-3`)	PAM	Off-target
eLoxP-1(pX330)	GTATGCTATACGAAGTTATT	AGG	
Chr 1	GTAGGGTAAAGGAAGTTATT	AGG	No
Chr 3	CTATGACATACTAAGTTATT	AGG	No
Chr 6	GTAT ACTGTTTGAAGTTATT	TGG	No
Chr 8	AAATGCTAACCGAAGTTATT	TGG	No
Chr 9	AAATGCTAAAGGAAGTTATT	TAG	No
Chr 10	AAATGCTTTAGGAAGTTATT	CGG	No
Chr 12	AAATGCTAAAAGAAGTTATT	CAG	No
Chr 13	GTAATGTATACAAAGTTATT	TGG	No
Chr 13	ATATTCTCTAAGAAGTTATT	AGG	No
Chr 18	TAATGCTAAAGGAAGTTATT	TGG	No

Table S4 Off-target analysis for CRISPR/SaCas9-*loxP* System **PCR primers**

Primer Name	Primer sequence (5`-3`)	Product(bp)	Anneaing(℃)
F17	GCATCACAGTCACAGCATTT	263bp	58
R17	GAAAGGGCAGACCTAGGATATAA		
F18	CATTAGGCTTTATCGACCTAGTGA	373bp	58
R18	CATGTCAATTTAGGCAGGGTTT		
F19	GTAGCCTCTAGCCACATGTTT	320bp	58
R19	TCACCACCTTTGGTGTTCTC		
F20	CCTGTTCCACTCTCCCTTTATAC	386bp	58
R20	ACAGAAGAGTAGACCTATTTGTAAG		
F21	CCTCCTGAATAGCTGGGATTAC	391bp	58
R21	CCCTCCCTGGTTAGAAGAAAC		
F22	GGCAGAATCTGAAAGTGGATACT	551bp	58
R22	GCCTCAATGAGCTCCCAAATA		
F23	AACCCAATTCCCATCAACTAAATC	446bp	58
R23	AGGGAAGAGTCATTGGTAAAGTC		
F24	GTGCCTGGCCAACATTTATC	459bp	58
R24	CCCACAAGAGTGGAGGATTATT		
F25	CTGTTGAGCATTTAATTTCGGTGA	436bp	58
R25	TCACACATGTCTTTGGAGATTGA		
F26	TCCAAGGTAGTGGCATTGATAC	410bp	58
R26	AAGAAAGGAGGGAACTCTTGATAC		

Analysis of off-target induced by CRISPR/SaCas9-*loxP* System

Chromosome	Target sequence (5`-3`)	PAM	Off-target
eLoxP-2(pX601)	TACATTATACGAAGTTATATT	AAGGGT	
Chr 4	TATATTATACAAACATATATT	AAGAAT	No
Chr 4	TTCATTATATGAAGTTCAATT	CAGGAT	No
Chr 7	CACTATCTACGAGGTTATATT	AAGTGA	No
Chr 7	ACTACTTTTAGAAGTTATATT	AAGGCC	No
Chr 8	TTCTTTATAAGAAGATATATT	CTGAGT	No
Chr 11	TACAATCTATGAAGTGATATT	TAGGAT	No
Chr 13	ACATTATACGATGTTACATT	ATATGA	No
Chr 19	TAAACCCAAAGAAGTTATATT	AAGATT	No
Chr X	TACTTTATATAAACTTATATT	TGGAAT	No
Chr X	TCACTTCAAGAATGTTATATT	AAGGGA	No