

TECHNICAL REPORT

An efficient and precise method for generating knockout cell lines based on CRISPR-Cas9 system

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Abstract

Although the efficiency and versatility of CRISPR-Cas9 system has been greatly improved over conventional genome editing methods such as zinc finger or TALEN, it is still time-consuming and labor-intensive for screening knockout/knock-in cell clones due to differences of the targeted location or efficiencies of guide RNAs (gRNAs). Here, we adapted a targeted knock-in strategy with CRISPR-Cas9 system and characterized the efficiency for generating single or double knockout cell lines. Specifically, a homology-arm based donor cassette consisting of genes encoding a fluorescence protein and antibiotic selection marker driven by a constitutive promoter was co-transfected with a gRNA expressing unit. Based on FACS sorting and antibiotic drug selection, positive cell clones were confirmed by genotyping and at the protein expression level. The results indicated that more than 70% of analyzed clones identified by cell sorting and selection were successfully targeted in both single and double knockout experiments. The procedure takes less than three weeks to obtain knockout cell lines. We believe that this methodology could be applicable and versatile in generating knockout cell clones with high efficiency in most cell lines.

KEYWORDS

CRISPR-Cas9, homologous recombination, knock-in, knockout, targeting efficiency

1 | INTRODUCTION

Targeted gene disruption has been widely used for deciphering gene functions in mammalian cell development. Compared with zinc-finger and TALEN genome editing

tools, CRISPR-Cas9 system has become the most widely used owing to its simplicity and high efficiency [1, 2]. Despite its apparent simplicity, the efficiency and specificity of genome editing can be impacted by technical parameters in whole procedure. Although CRISPR-Cas9 mediated gene knockout has been optimized in delivery process either by co-transfection of sgRNA and Cas9 expressing plasmids or transfection of a recombinant ribonucleoprotein complex [3], it is still time-consuming and labor-intensive for upstream gRNA optimization

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CBh, hybrid chicken β -actin promoter; EGFP, enhanced green fluorescent protein; gRNAs, guide RNAs; INDELS, insertions and deletions; KO, knockout; NHEJ, non-homologous end joining; PAM, protospacer-adjacent motif

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and downstream isolation of cell clones with targeted disruption. In order to achieve rapid screening of positive clones, Andras *et al* prescreened the gRNAs through integration of a “self-cleaving” GFP expression plasmid mediated by non-homologous end joining (NHEJ) which facilitates the targeting efficiency from the starting point [4]. To enrich the genetic modified cell clones, fluorescence proteins or antibiotic resistance genes are commonly fused to the Cas9 protein expressing cassette for selection after transfection. Alternatively, a separate fluorescence protein or antibiotic resistance gene cassette was co-transfected. For example, Branden *et al* enriched the targeted cells by co-transposition of *PiggyBac* based drug resistance cassette [5]. Although these methods improved and speed up the targeting efficiency to some extent, the biallelic gene knockouts have to be screened through intensive genotyping and characterization [6]. Furthermore, these methods cannot exclude those false positive clones due to the random integration. To reduce the random integration events and enhance targeting specificity, homology arm-based integration combined with CRISPR-Cas9 system has been used in mammalian cells for gene knockout either by insertion of foreign antibiotic cassette with splice acceptor into the intron or insertion of a selectable, promoterless knockout (KO) cassette at the coding sequence of a targeted active gene locus [6, 7]. However, the promoterless KO strategy may be unsuitable for those genes with weak or no expression. In addition, the efficiency of homology arm-based gene knockout has not been systematically characterized in previous studies.

Here, we adapted a targeted knock-in strategy to disrupt gene function based on CRISPR-Cas9 system and characterized the efficiency of targeting one or two genes simultaneously. Specifically, homology arm directed cassette consisting fluorescence protein and antibiotic resistance gene driven by constitutive promoter was provided as donor. The precise insertion of homology-arm directed cassette into the exon could terminate the transcription and lead to loss of function of targeted genes. Furthermore, the inserted cassette is often accompanied by an insertions and deletions (INDELS) mutation in another allele which circumventing the need for another allele mutation [8]. In order to exclude the potential effect of fluorescence protein and drug resistance gene in knockout cells, the inserted cassette flanked by two loxPs could be deleted with *Cre-loxP* system.

To test the efficiency of this knockout strategy, two separate experiments targeting one and two genes were performed separately. The first gene we chose is *fut8* gene which encodes α -1, 6-fucosyltransferase and catalyzes the transfer of fucose from GDP-fucose to the innermost N-acetylglucosamine (GlcNAc) of Fc oligosaccharides via α -1,6-linkage [9]. Fucosylation of antibody Fc core

PRACTICAL APPLICATION

The research we presented here could be time-saving and high efficient for studying gene function using knockout strategy based on CRISPR-Cas9 system. From our studies, both monoallelic and biallelic cell clones could be used for gene functional studies. Furthermore, the inserted cassette could be removed or modified with Cre-loxP system which provides researchers more flexibility.

region have been found to inhibit antibody function in antibody-dependent cell-mediated cytotoxicity (ADCC) which is an immune response induced primarily by natural killer cells against antibody binded targets [10]. The antibody produced from CHO cells with disruption of *fut8* enzyme was already proved to enhance ADCC activity 100-fold in previous studies [11–13]. To produce therapeutic antibody without fucosylation, we first disrupted *fut8* protein in CHO cells using this method. To test the KO efficiency of targeting multiple genes, we also simultaneously targeted two genes (Pravin and Snail) in 3T3 cells which were found to be related to cell migration in fibroblast in our laboratory (data not published). All the results indicated that above 70% of analyzed clones identified by FACS sorting and drug selection are correctly targeted in the genome indicating that this method can be applied to generate multiple knockout cell clones with high efficiency in most cell lines.

2 | MATERIALS AND METHODS

2.1 | Materials

CHO-K1 cell line and NIH-3T3 cell line was a gift from Dr. Chunhong Yu. Parvin and Snail antibody were purchased from CST Company (Cell Signaling Technology, Danvers, MA, USA). For molecular cloning, all primers are synthesized from GENEWIZ Company (Suzhou China). For mammalian cell culture, DMEM, F12K nutrient mixture, FBS are from Invitrogen (Carlsbad, CA, USA).

2.2 | Vector construction

In the donor cassette, the enhanced green fluorescent protein (EGFP) and blasticidin resistant gene was linked with T2A “self-cleaving” peptide and driven by a constitutive

hybrid chicken β -actin (CBh) promoter. Homology arms ranging from 800 to 1 kb were used for homologous recombination in donor cassettes. All the aforementioned cassettes were synthesized by Wuxi Qinglan Biotech., Ltd and cloned into the pMV plasmid.

2.3 | Cell culture and transfection

CHO-K1 cells were cultured with F12K nutrient mixture medium supplemented with 10% FBS and 1% v/v penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). When cells (in 1 well of a 12-well plate) reach about 60–70% confluence, 1 μ g fut8 knock-in cassette and 0.5 μ g PX330-gRNA were co-transfected with lipofectamine3000 reagent (Invitrogen, Carlsbad, CA, USA). After 24 hours, the cells were trypsinized and sorted into 96-well plate based on positiveEGFP expression. Then the culture medium were refreshed and supplemented with 6 μ g/mL blasticidin antibiotics for removal of transient transfection cell clones the next day. After one week of culture and selection, the survival clones were propagated for functional analysis.

NIH-3T3 cells were cultured with DMEM high glucose medium supplemented with 10% FBS and 1% v/v penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). For transfection of Parvin and Snail gene cassettes, 5×10^5 NIH-3T3 cells were seeded into one well of 12-well plate. After 24 h, 1 μ g of each Parvin and Snail gene cassette and 0.5 μ g of corresponding PX330-gRNA were co-transfected into the cells using lipofectamine3000 reagent. The next day, transfected cells were trypsinized and sorted into 96-well plate based on positive EGFP and mCherry expressions. After 24 h, the culture medium were refreshed and supplemented with 2 μ g/mL blasticidin antibiotics. After one week of culture and selection, the survival clones were propagated for functional analysis.

2.4 | Genotyping analysis

The propagated cells were trypsinized and washed twice with $1 \times$ PBS, genomic DNA was isolated using Genomic DNA extract kit (Invitrogen, Carlsbad, CA, USA) and resolved in 50 μ L TE buffer finally. Subsequently, 1 μ L DNA was used as template for PCR amplification. PCR was performed with 30 cycles of 94°C 30 s, 60°C 30 s, and 72°C 4 min with designed PCR primer pairs. The primers used in this paper are as following:

Fut8-gRNA-F1: CACCGTCAGACGCACTGACAAA-GT,
Fut8-gRNA-R1: AAACACTTTGTGTCAGTGCGTC TG-AC;

Parvin-gRNA-F1: CACCGTCGCCTCCGTCCCCGCA-AGA,
Parvin-gRNA-R1: AAACCTTTGCGGGACGGAGGC-GAC;
Snail-gRNA-F1: CACCAAGGACGACGGCGCCGGC-GA,
Snail-gRNA-R1: AAACCTCGCCGGCGCCGTCTGTTT;
Fut8-5'armF: TCTGTTGATTCCAGGTTCCC,
Fut8-3'flank R2: GTCTTACATTTGGCTGATAAGT-TTC;
Parvin-5'arm F: TAATTCCGCTTCCGTTTG,
Parvin-3'flank R: CCATCTTTGTTATTGGCTCA;
Snail -5'flank F: TGCATATCATCGCACTTTCTG,
Snail-3'arm R: TCTAACCCTGAGCCGTCTTC;

2.5 | LCA reactivity assay

Cells (1 well of 6-well plate, about 10^6 cells) were trypsinized and neutralized with culture medium, centrifuged and suspended with $1 \times$ PBS, then washed with 300 μ L $1 \times$ PBS containing 0.1% BSA. 2 μ g/mL Dylight649 (final concentration) labeled Lens culinaris agglutinin (LCA; Vector Laboratories, Burlingame, CA) was added into the cells and incubated for 30 min on ice. Then, the stained cells were analyzed with FACSCanto SORP (BD Biosciences, San Jose, CA).

2.6 | Image analysis and flow cytometry

Bright field and fluorescence pictures were taken from Nikon inverted fluorescence microscopy Ts2-FL (Nikon corporation, Tokyo, Japan) with $10 \times$ objective. Flow cytometry results were analyzed with BD FACSCanto SORP Analyzer, at least 10 000 events were recorded for analysis with FLOWJO software (Emerald Biotech Co., Ltd, Hangzhou, China). GFP fluorescence signal was detected by excitation with 488 blue laser and emission with 530/30 band pass filters.

2.7 | Western blot

Western blotting was performed as previously described [14]. Equal amounts (10–40 μ g per lane) of cell proteins were separated on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 5% non-fat powdered milk, followed by overnight incubation at 4°C with rabbit anti- α -Parvin (D7F9) XP

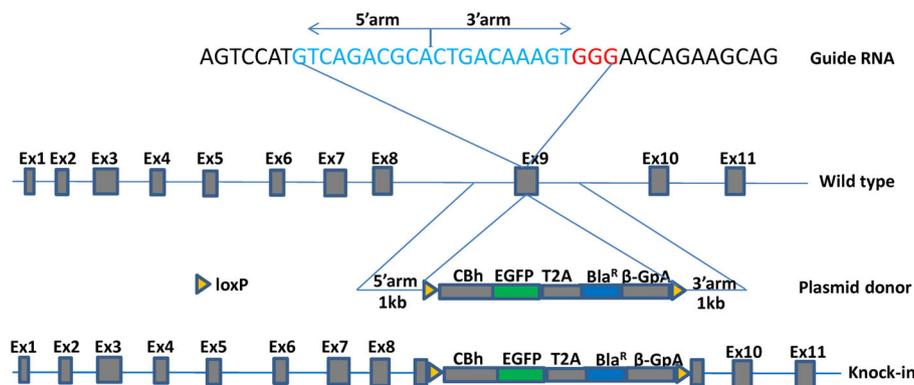


FIGURE 1 Schematic representation of Fut8-ko strategy based on homology arm directed cassette. The sgRNA-targeting sequence is labeled in blue and protospacer-adjacent motif (PAM) sequence are labeled in red; CBh, hybrid chicken β -actin promoter; EGFP, enhanced green fluorescence protein; β -GpA, β -globin polyA signal; Bla^R, blasticidinresistance gene

(CST, #8190,1:1000), rabbit anti-Snail (C15D3) (CST, #3879, 1:1000) antibodies. After washing and incubation with appropriate horseradish peroxidase-conjugated secondary anti-rabbit or mouse IgG antibodies (Jackson ImmunoResearch, #711-005-152 or #715-005-151, 1:10,000), blots were developed using an enhanced chemiluminescence kit (ECLKit, Bio-Rad) and then exposed to X-ray film (Fuji film, #super RX-N-C). The images were scanned using an imaging scanning system (EPSON Scan; L365).

3 | RESULTS

3.1 | Design and generation of fut8 gene knockout construct

Although NHEJ mediated gene knockout is easier designed, it is time-consuming and labor-intensive for isolation of correctly targeted knockout cell clones. To rapidly isolate the correctly targeted cell clones after transfection, homology arm directed donor cassettes were employed in our constructs. To enable efficient sorting and selection of positive clones after transfection, donor cassette comprising fluorescence protein and antibiotics resistant gene sequences were assembled together vis self-cleaving T2A peptide and driven by a constant CBh promoter. 1 kb of 5' and 3' homology arm were flanked at both ends of expression cassette (Figure 1). Such donor cassette will be inserted into the exons of gene of interest (GOI), in frame with the encode protein and lead to premature termination of target gene expression.

3.2 | Targeted disruption of fut8 gene function

To test the efficiency of this method, we first chose fut8 gene which encodes α -1, 6-fucosyltransferase and directs

fucose to antibody Fc region. The disruption of fut8 gene was often used to enhance antibody-dependent cellular cytotoxicity (ADCC) in CHO cell line. Since the active region of fut8 enzyme is located in exon9, a guide RNA from exon9 was chosen for disrupting fut8 gene function [15]. To pick the clones with gRNA directed knock-in cassette, EGFP positive cells were sorted into 96-well plate with flow cytometry (FACS Aria SORP) based on EGFP fluorescence protein expression 24 h after transfection (Figure 2A). To exclude the clones with transient transfection, cell medium was refreshed with 6 μ g/mL blasticidin antibiotics containing culture medium the next day. After one week of growth and selection, the survived cell clones were propagated. Fluorescent protein expressions were confirmed with inverted fluorescence microscopy (Figure 2B). In order to distinguish monoallelic or biallelic cassette integration rapidly, genotyping analysis was performed with extracted genomic DNA of ten clones. The PCR primers were designed with one in homology arm and the other outside of homology arm which can provide information whether one or both alleles were targeted. Results showed that three clones were double knockout and four clones were single copy deleted among ten analyzed cell clones (Figure 2C) which indicates that the targeted knockout efficiency is about 70%.

3.3 | Fucosylation detection and genetic stability analysis of Fut8 knockout cell clones

To evaluate the activity of α -1, 6 fucosylation in Fut8 disrupted clones, we analyzed the reactivity of fut8 knockout clones against LCA, which recognizes the α -1, 6-fucosylated trimannosyl-core structure of N-linked oligosaccharides. Flow cytometry analysis revealed that the LCA activity of disrupted fut8 cells was reduced by 100 fold over parental CHO cells (Figure 2D). This result is

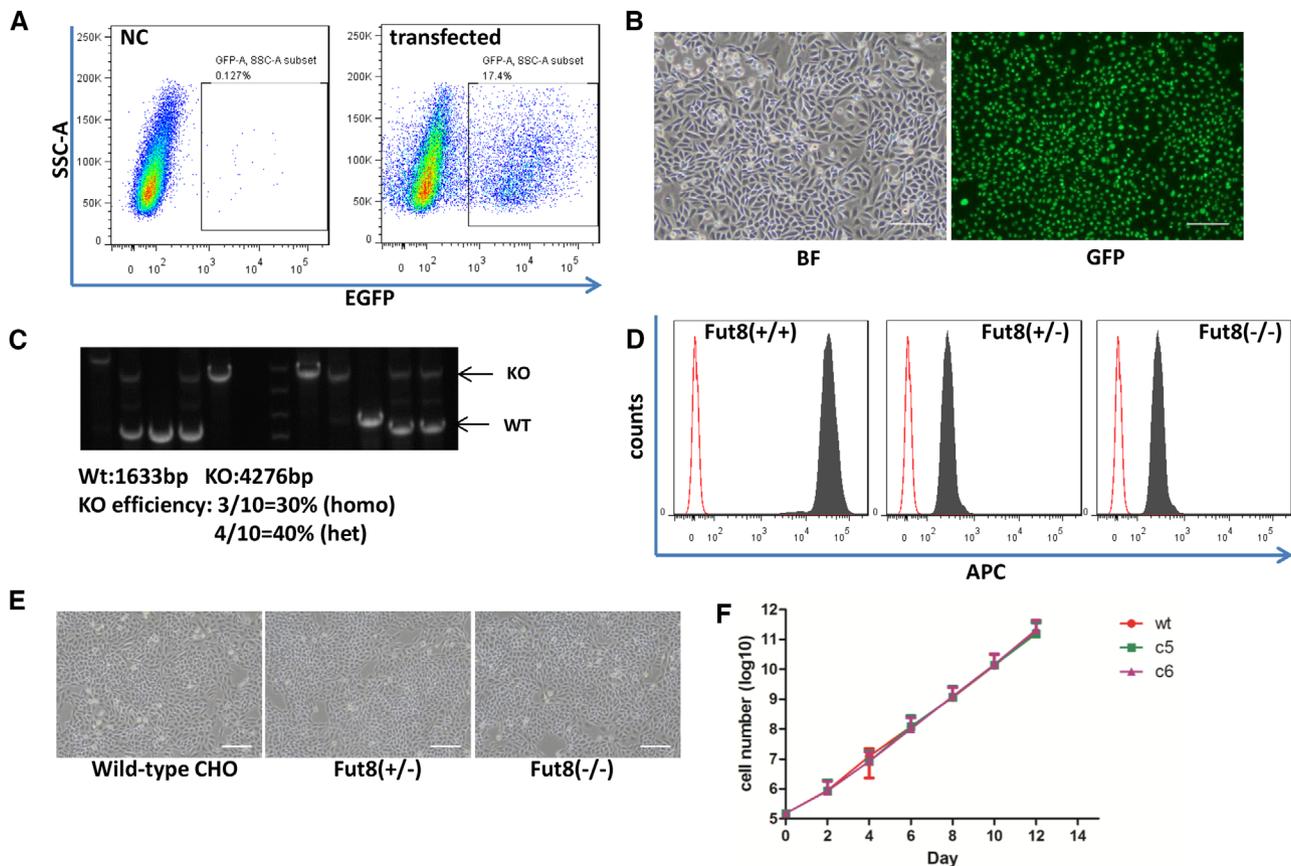


FIGURE 2 Genotyping and characterization of *Fut8* disrupted cell clones. (A) Flow cytometry analysis of transfected CHO cells with *Fut8*-KO donor cassette. NC represents untransfected CHO cells as negative control. (B) The fluorescence picture of EGFP positive cell clones after FACS sorting, the scale bar is 100 μm. (C) Genotyping analysis of ten EGFP positive and blasticidin survived cell clones. The length of wild type and knockout PCR products is 1.6 and 4.3 kb, separately. Het represents mono-allelic mutation and homo represents biallelic mutation. (D) Flow cytometry analysis of LCA activity of *fut8*-ko cell clones. The red histogram represents the wild type CHO cells without LCA treatments. *Fut8*(+/+) represents wild type CHO cells with LCA treatment for 30 min, *Fut8*(+/-) and *Fut8*(-/-) represents the *fut8*-ko cells with LCA treatment for 30 min. The mean APC channel value of wild type CHO versus *Fut8*-KO cells is 35000:320. (E) The morphology of wild type and *fut8*-ko cells, the scale bar is 100 μm. (F) Cell growth of wild type and *fut8*-ko cells. The cell numbers were calculated every two days during each subculture and identical numbers of cells were seeded for continuous passaging. C5 and C6 represent two cell clones with *Fut8* mutation

consistent with the data described in previous reports [11, 15]. Furthermore, the LCA activity was reduced similarly in both monoallelic and biallelic targeted clones indicating that another copy of *fut8* gene was also mutated in monoallelic targeted cells and this phenomenon has been reported in previous studies [16]. To evaluate whether disrupted *fut8* gene has impact on cell morphology and growth rate, wild type CHO and *fut8* disrupted cell clones were captured with inverted microscopy and passaged continuously for 10 days, and results indicate that there are no differences between wild type and KO cell clones on cell morphology (Figure 2E). Furthermore, *Fut8* disrupted cells are capable of propagating exponentially with compatible growth rates compared with wild type CHO cells (Figure 2F). These results indicate that the *Fut8* gene has been successfully knocked out.

3.4 | Targeted disruption of Parvin and Snail genes and functional characterization

In order to test the efficiency of multiple gene knockout with our approach, Parvin and Snail genes which were previously found to be related to cell migration in fibroblast (data not published) were chosen for targeting. The strategy adopted was similar to the one used to generate *fut8* gene knockout. Two different sets of fluorescence proteins and antibiotics resistant genes were chosen for sorting and selection. 800 bp of 5' and 3' homology arms were used for homologous recombination (Figure 3A and B). Then all the cassettes were commercially synthesized.

Compared with single gene transfection efficiency, the transfection efficiency for double genes was greatly reduced (Figure 4A). However, it has no impact on the

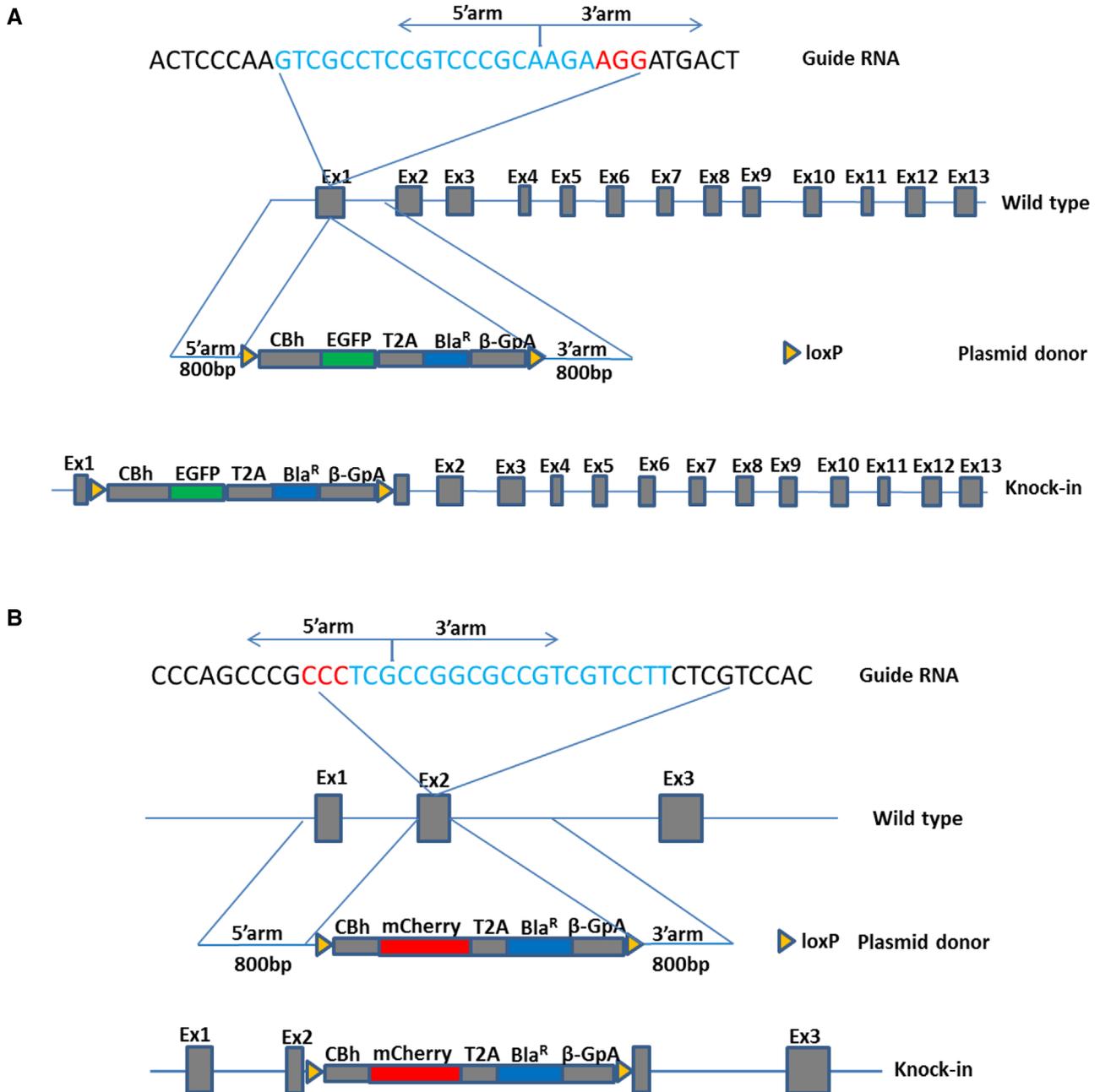


FIGURE 3 Schematic representation of Parvin and Snail knockout strategy based on homology arm directed cassette. The sgRNA-targeting sequence is labeled in blue and protospacer-adjacent motif (PAM) sequence are labeled in red; CBh, hybrid chicken β -actin promoter; EGFP, enhanced green fluorescence protein; β -GpA, β -globin polyA signal; Bla^R, blasticidin-resistance gene

positive clone selection based on FACS cell sorting. Twenty-four hours after transfection, GFP and mCherry positive cell clones were sorted into 96-well plate with a FACS Aria SORP sorter. The next day, blasticidin antibiotics (2 μ g/mL) was added into the culture medium for the maintenance and selection. After one week of culture and antibiotics selection, the survival cell clones were propagated and imaged with fluorescence microscopy which shows strong GFP and mCherry expression (Figure 4B). In order to check out whether the survival

clones were properly inserted into the correct location in the genome, the genome DNA of ten survived clones were extracted and genotyped with a pair of designed primers for Parvin and Snail, respectively. The results showed that two were double knockout for Parvin and four clones were double knockout for Snail. The most remaining clones were heterozygous insertion (Figure 4C). This suggests that the efficiency for double gene knockout was compatible with single gene disruption. We also detected the protein expression levels of disrupted Parvin and Snail clones.

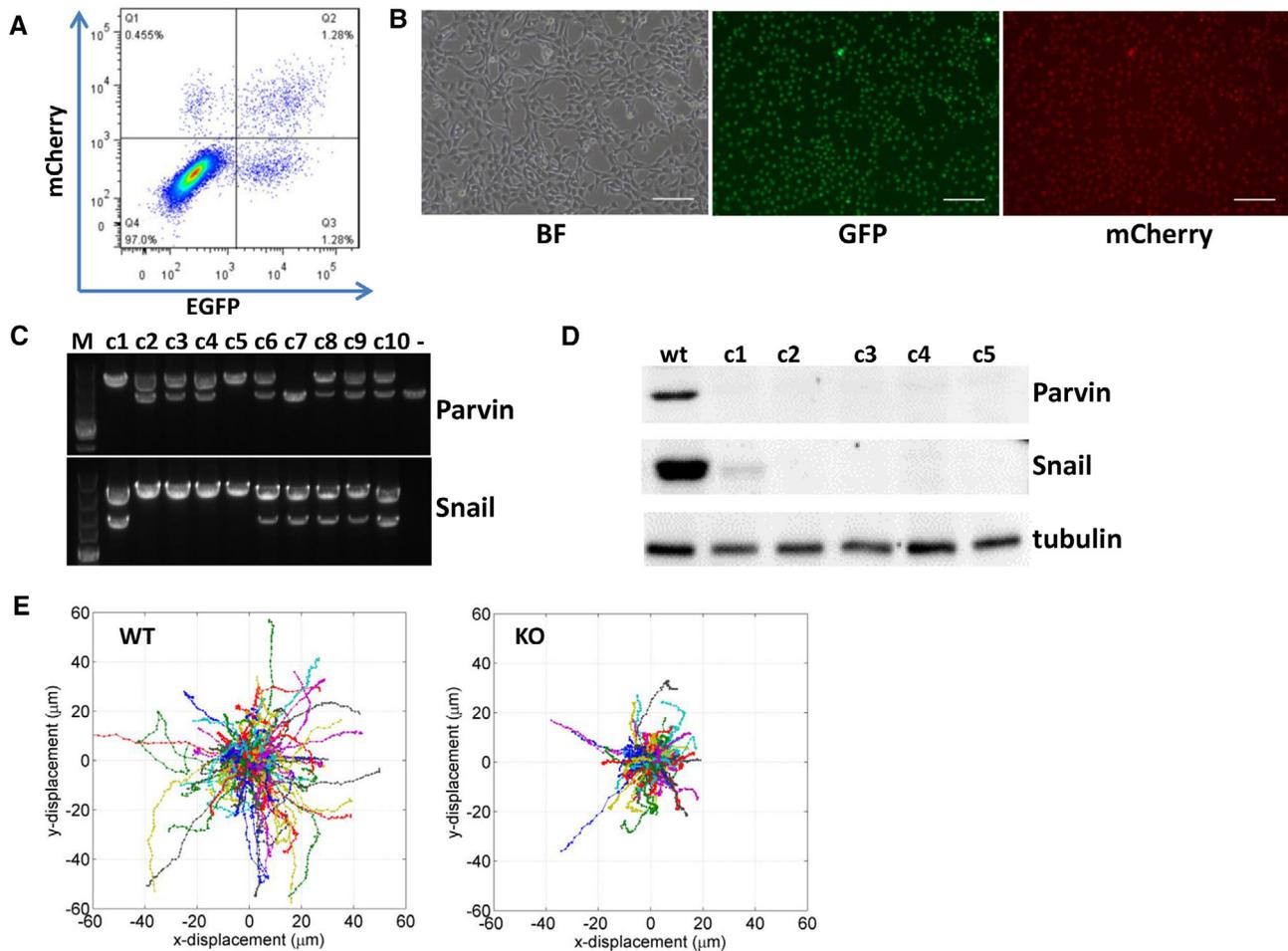


FIGURE 4 Characterization of Parvin and Snail double knockout cell clones. (A) Flow cytometry analysis of parva and snail transfected cells. Q2 represents EGFP and mCherry double positive cells. (B) The fluorescence picture of EGFP and mCherry positive cell clones, the scale bar is 100 μm . (C) Genotyping analysis of ten EGFP and mCherry double positive as well as blasticidin survived cell clones. (D) Western blotting of C1-C5 clones for parva and snail protein expression. (E) Migration plots of wild-type (WT) and Parvin/Snail double knockout (KO) 3T3 fibroblasts by tracking 266 cells each from 400 to 700 min after seeding. The starting point of each trajectory was translocated normalized to $x = 0$ and $y = 0$ to exhibit motility. Both cells were cultured in serum free DMEM culture medium

Western blotting confirmed that Snail protein expression was undetected for clone 2-5 which was confirmed biallelic mutation from genotyping analysis, only clone 1 with monoallelic mutation showed very weak protein expression. For Parvin gene of detected five clones, all the protein expression was also diminished in both mono and biallelic integrated clones (Figure 4D). It has been reported that the foreign cassette insertion into one allele is commonly accompanied by an out-of-frame indel mutation in other allele [6, 16]. This result is consistent with previous reports and indicates that this method has high efficiency for multiple gene knockouts and both monoallelic and biallelic knockout clones could be used for functional analysis.

To check whether Parvin and Snail knockout has impact on cell motility, cells with Parvin and Snail double knockout were tracked using live cell fluorescent microscopy

and in house cell tracking algorithm [17]. Particularly, cells with Parvin and Snail knockout were cultured without serum. The wild type 3T3 fibroblast was used as control. After seeding for 30 minutes, both knockout and wild type cells were captured with inverted Nikon *Ti-E* microscope for total time of 700 min and time interval of 5 min. As shown in Figure 4E, of tracked 266 cells in both knockout and wild type 3T3 cells from 400 to 700 min, double knockout 3T3 cells migrate significantly slower than wild type cells. This result confirmed the functions of Parvin and Snail in regulation of cell motility.

4 | DISCUSSION

Here, we characterized the knockout targeting efficiency based on homology-arm directed integration combined

TABLE 1 Gene targeting efficiency data

Target gene/marker	% Fluorescence protein positive cells	Antibiotics resistant clone number/96 cells	Analyzed clone number	Targeted clone number
Fut8 (EGFP)	17.4%	24	10	7
Parvin+Snail (EGFP+mCherry)	1.28%	10	10	7

with CRISPR-Cas9 system. Two sets of genes were selected for testing this method. Results showed that this homology-arm based method has very high targeting efficiency in generating knockout cell clones, and all the clones with mono or biallelic mutation could be used for functional analysis.

The normal strategy for creating knockout cell line with CRISPR-Cas9 system is based on its non-homologous end joining mechanism, which often lead to INDELS upon double strand breaks. Although it is very convenient for preliminary molecular cloning, it is time-consuming and labor-intensive for screening positive targeted cell clones owing to gRNA activity variation and targeted genome location environment. In contrast, homology-arm based genomic insertion can be controlled precisely. In order to simplify the screening process after transfection, we introduced fluorescence protein and drug selection marker at the knock-in donor cassette. Based on FACS cell sorting and drug selection, the whole procedure for generating knockout cell lines is less within three weeks. Furthermore, the precise knock-in targeting efficiency is up to 70% both for single gene or double gene targeting indicating that this strategy is very high efficient (the targeting efficiency is summarized in Table 1). For targeting two genes simultaneously, we used the same antibiotic resistance gene for selection, it could be envisioned that the targeting efficiency could be further improved if two different antibiotic selection genes were introduced for enriching the survival cell clones. Most importantly, donor cassette flanked by two loxPs could be removed optionally which provides more flexibility.

In previous studies, the homology arm length used varies from 30 bp to several kilo bases (kb) [18–20]. And it was shown that the targeting efficiency increases with longer homology arm [21]. The homology arms in our designed cassette range from 800 bp-1 kb, and the fluorescence and drug resistance gene cassette which is established in our lab is about 2.5 kb. Although we can amplify the homology arm and cloned into both ends of the selection cassette with conventional method which is cost-effective, however, it may prolong the whole procedure due to the cloning steps. Furthermore, the cost of gene synthesis is much lower with the development of sequencing techniques which enables researchers to shorten and speed up the molecular cloning.

During the protein level identification, we found that the protein encoded by targeted gene is also diminished in monoallelic integrated cell clones indicating that another gene copy is also mutated. And this phenomenon is also observed in previous researches [7, 16] which avoid the second round targeting for creating biallelic mutation. Aleksandra *et al* adopted similar methodology for genome editing in which they designed a promoterless antibiotic resistance gene coding cassette with flanking homology arms in both ends [6]. However, the selection marker expression level has to be dependent on the endogenous gene promoter which may mask the screening efficiency if the endogenous promoter derived gene expression is too weak. In Parvin and Snail double targeted cell clones, we found that one clone with monoallelic mutation still show weak Snail protein expression although the level is weak. Since we inserted the donor cassette in frame with endogenous protein sequence, the remaining protein expression may be transcribed from an in frame indel mutation.

In summary, the protocol we presented here could be time-saving and high efficient for studying gene function using knockout strategy based on CRISPR-Cas9 system. From our studies, both monoallelic and biallelic cell clones could be used for gene functional studies. Furthermore, the inserted cassette could be removed or modified with Cre-loxP system which provides researchers more flexibility.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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