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ORIGINAL RESEARCH PAPER

A suicidal zinc finger nuclease expression coupled with a surrogate reporter for efficient genome engineering

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Abstract Genome editing with engineered nucleases, such as zinc-finger nucleases (ZFNs) and TALE nucleases, remains confronted with a high risk of cellular toxicity induced by off-targeting. Here we describe the construction of a suicidal nuclease expression vector in which a pair of ZFNs genes were flanked of its target sites. To further enrich the targeted cells, the suicidal ZFN expression cassette was also inserted within an eGFP reporter, to disrupt the ORF of eGFP gene. ZFN-associated toxicity was reduced by ~40 % with this new system, and the activities of ZFNs were ~4.5 % lower. We conclude that using this new suicidal ZFN expression and surrogate reporter system represents an improvement for genomic editing by reducing toxicity and allowing easy detection of edited cells by eGFP analysis.

Cunfang Zhang and Kun Xu have contributed equally to this work.

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Introduction

Various endonucleases can be engineered to induce double-strand-breaks (DSBs) in target DNA sequences of interest. In the absence of donor DNA, these DSBs are spontaneously repaired by Non-Homologous-End-Joining (NHEJ) resulting in micro-insertions or micro-deletions. However, in the presence of appropriate donor DNAs, these DSBs can be repaired by homologous recombination (Lieber 2010; Moynahan and Jasin 2010). These specifically introduced DSBs can increase the efficiency of gene modification dramatically. Thus, engineered endonucleases are powerful tools to enable precise genome engineering to generate modifications such as point mutations, accurate insertions and deletions, and conditional knock-outs or knock-ins.

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Engineered zinc-finger nucleases (ZFNs) and TALE nucleases (TALENs) have been widely used in a variety of species and cell types. ZFN-mediated gene editing frequencies can reach up to 50 % in human stem cells (Lombardo et al. 2007). However, the potential side-effects of ZFNs system, such as toxicity, has curtailed the widespread use of this technology. In addition, the efficiencies of ZFN-based approaches are low in pigs, cattle, and other domestic animals cells. A surrogate reporter system designed by Kim et al. (2011) for enrichment of gene-modified cells may help to solve these problems. In this system, the plasmid contains a mRFP–eGFP fusion protein, and the ZFN target site is between the encoding mRFP and eGFP, which breaks the ORF of eGFP. In such system, green fluorescence could only be detected when the cognate ZFN have cleaved the target site in the reporter plasmid as well as the genome. Moreover, such system could also confirm the activity of ZFNs. However, this system is limited in that only one-third of the modified reporter plasmids in principle generated reconstituted eGFP based on restoration of the correct ORF, which could not reflect wholly the activity of ZFNs. In addition, ZFNs are continuously expressed and the persistent ZFNs may cleavage at further off-target sites in the genome, which may generate higher cytotoxicity.

In this study, we have created a new ZFNs system to overcome the limitation of previous system (Kim et al. (2011). In brief, our system contains mRFP–eGFP fusion protein, ZFN expression cassette and the ZFN target site between the encoding mRFP and eGFP, all of which breaks the ORF of eGFP. Based on our new system, three significant improvements were achieved: (i) the reporter eGFP has greater efficiency and accuracy. (ii) the toxicity is lower; and (iii) Moreover, other engineered nucleases could be used in this system as well.

Materials and methods

Expression vectors

pST1374-LZFN(pLZFN)and pST1374-RZFN(pRZFN) are ZFNs expression vectors, which target the sheep *MSTN* gene. Plasmids pDsRed1-C1 and pEGFP-C1 were obtained from Clontech, and plasmid pcDNA3-mRFP was obtained from Addgene.

Construction of reporter vectors

A gene encoding eGFP was divided into two segments, which were separated by a stop codon and an *MSTN* ZFN target site. Both eGFP segments eGFPL and eGFPR, sharing a 0.2 kb homology region, were amplified from pEGFP-C1 using the primers GFPL-F/GFPL-R and GFPR-F/GFPR-R described in Supplementary Table 1. The two amplified fragments were cloned into pDsRed1-C1 plasmid using *Bgl*II/*Sal*I and *Bam*HI/*Xba*I (NEB), respectively. The resulting vector was named pDG. The *MSTN* ZFN target site was generated by directly annealing oligonucleotides MBS-F and MBS-R described in Supplementary Table 1, and cloned into pDG plasmid bewteen *Bam*HI/*Not*I sites, resulting in the reporter plasmid pDGS. Transfection of pDGS into 293T cells demonstrated that DsRed expression was low. The RFP sequence was amplified from pcDNA3-mRFP and used to replace the DsRed sequence in pDGS, and this plasmid was designated as pRGS. The *MSTN* ZFNs expression cassette (PGK promoter-Left ZFN-T2A-Right ZFN-BGHpA) was cloned into the pRGS plasmid between the eGFPL sequence and *MSTN* ZFNs target site to achieve the first suicidal reporter plasmid pRGZS, which contains one ZFNs target site. Another *MSTN* ZFN target site was inserted into the pRGZS plasmid between the eGFPL sequence and the *MSTN* ZFNs expression cassette. This final suicidal reporter plasmid contains two ZFN target sites and was named pRGSZS. The sequences of all primers used to construct the pRGSZS vector are listed in Supplementary Table 1.

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 10 % (w/v) fetal bovine serum at 37 °C and 5 % CO_2. About 10^4 HEK293T cells were seeded in 0.5 ml culture medium into each well of a 24-well plate and cells were incubated for 24 h before transfection. For each sample, three duplicates were performed. Culture medium was replaced 1 h prior to transfection and transfection mix was prepared in a 1.5 ml microfuge tube as follows: mixing 1 μg DNA with 47 μl Opti-MEM (Sigma), 2.5 μl Lipofectamine 2000 (Invitrogen) together with 47.5 μl Opti-MEM.

The solution was incubated for 25 min at room temperature, and added to the prepared cells. Cells were maintained in a 5 % CO_2 atmosphere for 24 h.

ZFN activity assay

Three different vector combinations were transfected in HEK293T cells: pRGS alone, a mixture of pRGS, pLZFN and pRZFN (4:3:3, by vol.), and suicidal reporter plasmids pRGZS or pRGSZS. Cells were observed at 24, 48 and 72 h after transfection under the fluorescence microscope, respectively. After 72 h transfection, 30,000 cells were counted and evaluated the number of RFP Express (REx)-positive cells and eGFP Express (GEx)-positive cells by flow cytometry in each group. The transfection efficiency was calculated based on percentage of REx-positive cells, and the ZFN-activity was also calculated based on GEx-positive cells.

Zinc finger nuclease (ZFN)-associated toxicity assay

A negative control group of HEK293T cells transfected with 1 μg DNA (400 ng pRGS and 600 ng pST1374), two groups of ZFN treated HEK293T cells transfected with 1 μg DNA (400 ng pRGS, 300 ng pLZFN, and 300 ng pRZFN) and a final group transfected with 1 μg pRGSZS were used in these assays. After 30 h transfection, 30,000 cells were counted and the number of REx-positive cells by flow cytometry was evaluated. The remaining cells were placed in continuous culture. Five days later, the remaining cells were harvested and analyzed by flow cytometry as described above. The relative cell survival rate is calculated based on the ratio of REx-positive rate between 5 days and 30 h.

Results

Design and construction of a suicidal reporter system

In our new designed suicidal system, the expressed ZFNs in cells could cleave their target sites in the reporter plasmid. Subsequently, the *eGFP* gene would be repaired by HR, and the two ZFN target sites as well as the ZFN expression cassette would be removed (Fig. 1). In the process of plasmid construction, we found that DsRed-expression of the pDGS plasmid in HEK293T cells was low and similar phenomena were also observed with DsRed-eGFP fusion protein expression vectors (data not shown). To overcome this, RFP was used to replace DsRed and the expression level of this new vector is quite high (Fig. 2). The frequency of GEx-postive HEK293T cells was slightly lower comparing pRGZS transfection with a mixture of pRGS, pLZFN and pRZFN transfection; over 18 % of cells had undergone cleavage and homologous recombination (Fig. 2). Perez et al. (2005) reported that the presence of an

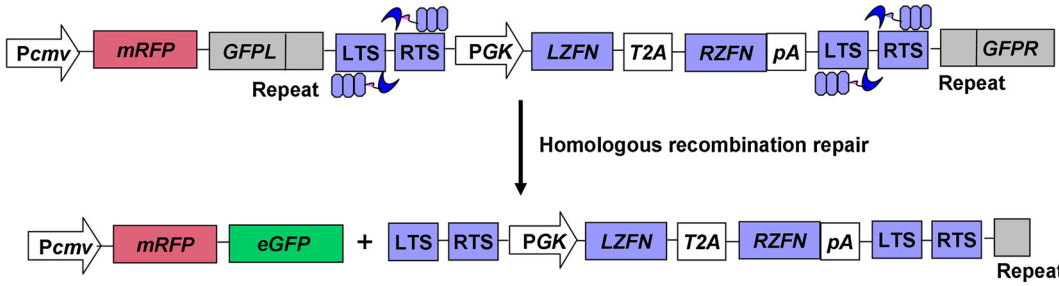

Fig. 1 Schematic representation of suicidal reporter system. The suicidal reporter vector is composed of one complete mRFP, two shortcut eGFP segments with a 200 bp repeat sequence, one ZFN expression cassette and two ZFN targeted sites (each composed of the left of the half target site, shorten as LTS, and right half of the target site, shorten as RTS). In this system, the ORF of eGFP is disrupted by the intervening sequences between the repeats. The expression of RFP, but not eGFP, will be detected whether cleavage occurs or not. When ZFNs are present and DSBs are introduced at both target sites, the eGFP gene will be repaired by homologous recombination and eGFP expression will be detected. Accordingly, detection of eGFP expression allows the identification of specific cleavage of the target sites by the ZFNs or other engineered-nucleases. In addition, self-cleavage causes the removal of the ZFN cassette from the suicidal reporter vector, abrogating ZFN expression and reducing toxicity associated with continuous expression of ZFNs

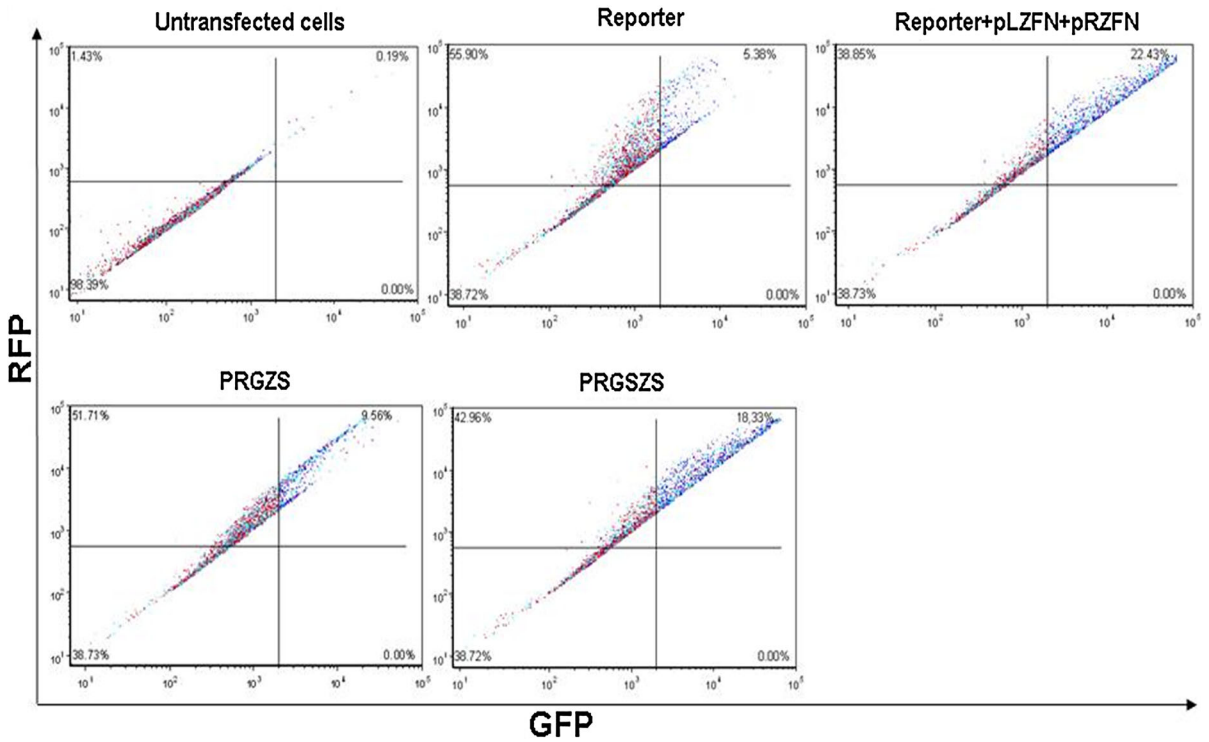

Fig. 2 Efficiency of RFP and eGFP expression in 293T cells transfected with a suicidal ZFN reporter system. Cells were transfected with a reporter plasmid and ZFN encoding plasmid or the suicidal reporter plasmid. 293T cells were subjected to flow cytometry 72 h after transfection. Untransfected cells and cells transfected with reporter plasmid were used as negative controls. Cells transfected with reporter, pLZFN and pRZFN plasmids, were used as positive controls. In the presence of the reporter only, eGFP expression was detected in approx. 5 % of cells, presumably because of spontaneous homologous recombination between two eGFP repeats

intervening sequence between gene duplications affects the efficiency of HR repair; however, our result strongly suggests efficient HR can be restored by positioning cleavage sites at both ends of the intervening sequence (Fig. 2). The reason of our higher efficiency of HR is we had used two eGFP homology arms (3 kb away)in our vector.

Analysis of ZFN activity

Compared with untransfected cells, HEK293T cells transfected with reporter plasmids exhibit low background expression of eGFP, which probably results from spontaneous homologous recombination between the homologous arms of eGFP. As shown in Fig. 2, co-transfection significantly increased eGFP positive expression. Co-transfection of the reporter and ZFNs boosted positive expression from 5.4 % (with only repoter vector) to 22.3 %. Also the positive expression increased from 9.6 % (with pRGZS) to 18.3 % (with pRGSZS). Following the same strategy described by Kim et al. (2011), the pRGS reporter vector was used to detect the activity of the assembled MSTN ZFNs in sheep fetus fibroblasts and to enrich endogenous gene-modified sheep fetus fibroblasts. The MSTN ZFNs were found to accurately target the *MSTN* gene of sheep as determined by PCR with MSTN-F/MSTN-R (Table 1) and sequencing in Fig. 3 (Zhang et al. 2014). Therefore, the pRGS reporter also could be used to identify the activity of screened and assembled ZFNs by various methods before its application in target cells.

Quantification of over-expression of ZFN-associated cytotoxicity

Although the pRGS reporter can be used for accurate and efficient gene targeting, the possible toxicity induced by continuous expression of ZFNs and off-target cleavage remains. We assessed the effects of

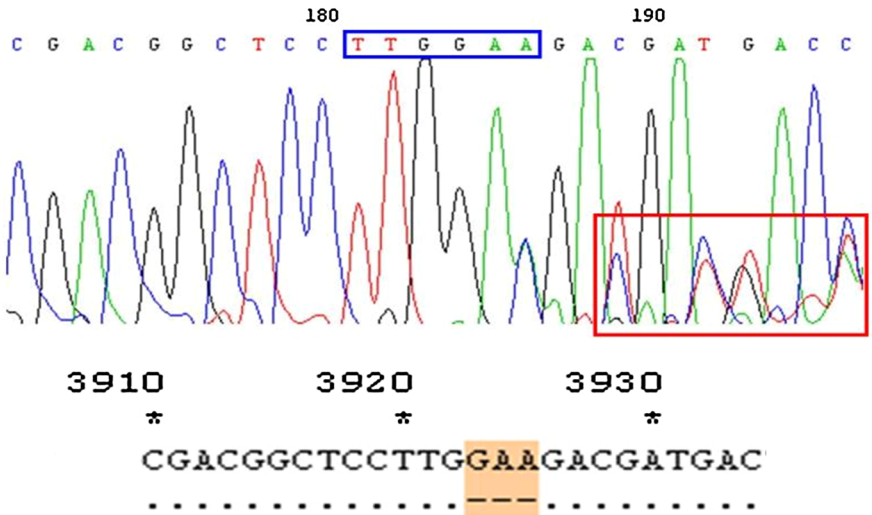

Fig. 3 Detection of ZFN induced genetic modification in sheep fibroblast cells. To detect mutations induced by the ZFN pair in sorted cells, PCR products of the sorted cells were sequenced directly. Usually, the modification of the DSBs near the cutting sites will be different in the two homologous chromosomes of one cell. In our experiment, 3 bp nucleotides were deleted from just one chromosome and no nucleotide deletion happened in the other chromosome, then specific MSTN-ZFN cleavage was verified by the presence of double peaks (*red box*) near the ZFNs cutting site (*blue box*). A 3 bp deletion in the cutting site was confirmed by comparing the MSTN sequences of sorted cells with wild sheep gene

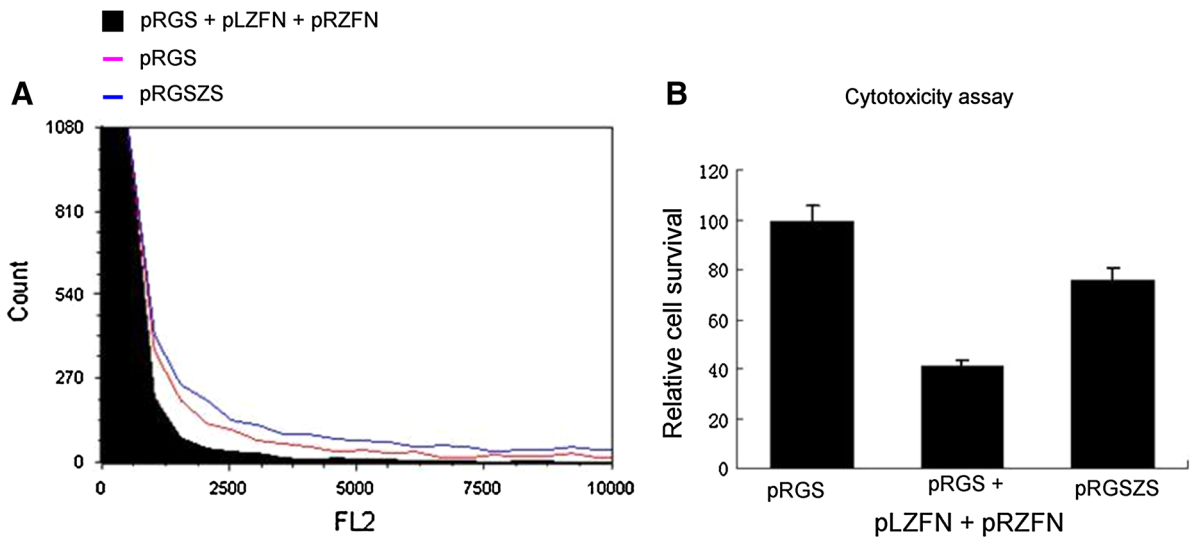

Fig. 4 Estimation of ZFN-associated toxicity. 30 h and 5 days after transfection, REx-positve cells were counted by flow cytometry in three groups. The first group was transfected with pRGS plasmid, the second with pRGS, pLZFN and pRZFN plasmids, and the third with pRGSZS plasmid. **a** While the efficiency of RFP expression was similar in each of the three groups 30 h after transfection, REx-positve cells transfected with pRGS, pLZFN and pRZFN plasmids were less than cells transfected with pRGSZS plasmid or cells transfected with pRGS plasmid. **b** Depiction of relative cell survival rate under the various experimental conditions. Cells transfected with the pRGS plasmid was considered to be non-toxic as it did not express ZFN; cells co-transfected with pRGS, pLZFN and pRZFN and these constitutively expressed ZFNs over the course of transfection; cells transfected with pRGSZS, in which ZFN mediated self-cleavage extinguished ZFN expression. Relative cells survival rates increased approx. twofold using the suicidal ZFN system

self-targeted cleavage on toxicity using the suicidal reporter plasmid pRGSZs according to Cornu and Cathomen (2010). The REx-positive cells with flow cytometry for cells transfected with pRGS, pRGS, pLZFN and pRZFN or pRGSZS at 30 h and 5 days were quantified after transfection. Data was analyzed by the Flow Express 4 software and it was turned out that the death rate of cells transfected with pRGS, pLZFN and pRZFN plasmids was higher than transfected with pRGSZS plasmid only (Fig. 4a). At the same time, the relative cell survival rate decreased from 80 (only pRGSZS transfection) to 40 % (co-transfection) (Fig. 4b). We concluded that the suicidal ZFN expression and a surrogate reporter system could reduce the ZFN-associated toxicity and nearly doubled the survival frequency. This probably results from self-cleavage of the pRGSZS plasmid, which showed subsequent ZFN expression.

Discussion

Engineered nucleases, such as zinc-finger nucleases (ZFNs) and TALE nucleases (TALENs), are powerful tools for genome engineering in both animals and plants. They have been used for endogenous gene disruption, targeted gene addition and chromosomal rearrangements in either cells or organisms ZFNs significantly increase the rate of gene targeting in a wide variety of experimental system that previously were not amenable to genome manipulation by homologous recombination (Porteus and Baltimore 2003; Bibikova et al. 2003; Urnov et al. 2005; Bibikova et al. 2002; Meng et al. 2008). One of the biggest blocks for the widespread application of ZFNs in genome engineering is the concern of off-target effects (Porteus and Baltimore 2003; Bibikova et al. 2002; Porteus 2006; Pruett-Miller et al. 2008). Several strategies to minimize engineered nuclease toxicity have been tried by either increasing the specificity of ZFNs or modifications of the nuclease (*fok1*) domain (Pruett-Miller et al. 2008;). For example, several studies have shown that increasing the amount of transfected ZFN expression plasmids can improve the efficiency of gene targeting (Pruett-Miller et al. 2008; Beumer et al. 2006). However, constitutive expression or high levels of ZFN protein could lead to high toxicity without increasing targeting rates (Bibikova et al. 2002). Additionally, high levels of ZFN expression caused abnormal developmental mutations in whole organisms such as flies (Bibikova et al. 2002; Beumer et al. 2006) and zebrafish (Meng et al. 2008). Therefore, regulation of ZFN expression could be an alternative strategy to attenuate ZFN toxicity. In this study, we have shown that a self-inactivating expression of ZFN coupled with a surrogate reporter gene not only significantly attenuated cellular toxicity induced by ZFN off-target cleavage, but also allowed rapid enrichment of ZFN modified cells by detection of eGFP expression. This novel design of ZFN increases cell surviving rate dramatically.

To regulate ZFN expression on transcriptional level, Silanskas et al. (2012) fused a ubiquitin moiety or FKBP12 domain to the *N*-terminus of ZFN respectively. Their strategies involved creating ZFNs with shortened half-lives and then regulating protein levels with small molecules. These alternative strategies maintained high rates of ZFN-mediated gene targeting while low ZFN toxicity. However, application of proteasome inhibitors in genome modification was complicated in the system. Our strategy of reducing ZFN toxicity is to terminate ZFN expression after ZFN cleaves its target sequence. Porteus and Baltimore (2003) provided evidence that maximal DSB-mediated gene targeting occurs within 60 h of transfection of ZFN expression plasmids, and this is consistent with our observation that the suicidal ZFN expression efficiently induces gene targeting after 72 h transfection. In addition to the regulation of ZFN expression via a suicidal expression designing, we also coupled a surrogate reporter gene with self-inactivating ZFN expression system. The ZFN expression cassette flanked by its target sequences was inserted in the middle of eGFP, which results in disruption of the ORF of the eGFP. If the ZFNs are functional, it first terminates its expression by cutting out its expression cassette from the vector, and then lead to restoration of eGFP open reading frame. Thus, the expression of eGFP protein could be an indicator of ZFN activity.

The application of programmable nuclease in genome engineering is also hampered by the lacking of good selection system. Without a selection marker, a large amount of work is needed. Kim et al. (2011) have designed a surrogate reporter system by inserting a ZFN target site between the DNA sequences encoding mRFP and eGFP. In this strategy, if a double strand break on the vector is generated by the ZFN, only one-third of the reporter plasmids could be

generated in-frame fusion eGFP gene; the reason is that nonhomologous end-joining-mediated deletion and insertion formation are random processes. We improved this surrogate system by adding 200 bp homologous sequence to the ZFN target site, where the double-strand break repair induced by ZFN could be repaired via homologous recombination.

TALEN and CRISPR have been developed to generate much greater target efficiency. However, off-targeting still happens when using these enzymes in genome editing (Wu et al. 2013). In consideration of the similar working mechanism of these artificial enzymes, it would be reasonable to believe that this suicidal system can be transferred easily to TALEN and CRISPR by replacement the ZFN coding gene with TALEN or CRISPR. Our suicidal reporter system is feasible and could also be used to identify the activity of ZFNs, TALENs or other engineered nucleases for testing purposes. It can also can be used to enrich mutated cells. Importantly, the use of self-limited expression by cleavage of the expression vector increased the cell survival rate dramatically. We conclude that our new system would be an alternative reporter system to edit genomic DNA with high efficiency, low cytotoxicity and the ability to enrich gene-modified cells.

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Supporting information Supplementary Table 1—Primers of construction suicidal reporter vector

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