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Targeted disruption of the sheep *MSTN* gene by engineered zinc-finger nucleases

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Abstract Prior to the development of zinc-finger nuclease technology, genetic manipulation by gene targeting achieved limited success in mammals, with the exception of mice and rat. Although ZFNs demonstrated highly effective gene targeted disruption in various model organisms, the activity of ZFNs in large domestic animals may be very low, and the probability of identifying ZFN-mediated positive targeted disruption events is small. In this paper, we used the context-dependent assembly method to synthesize two pairs of ZFNs targeted to the sheep *MSTN* gene. We verified the activity of these ZFNs using an mRFP-MBS-eGFP dual-fluorescence reporter system in HEK293T cells and, according to the expression level of eGFP, we obtained a pair of ZFNs that can recognize and cut the targeted *MSTN* site in the reporter vector. The activity of ZFN was increased by cold stimulation at 30 °C and by mutant the wildtype *Fok*I in ZFN with its counterpart Sharkeys. Finally, the ZF-Sharkeys and reporter vector were cotransfected into sheep fetal fibroblasts and two *MSTN* mutant cell lines, identified by flow cytometry and sequencing, were obtained.

Keywords Gene knockout · *MSTN* · Sheep · ZFN

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Introduction

Targeted gene modification is a powerful technique for investigating gene function, producing models of human disease and breeding animals with desired traits. It also holds much promise for the therapy of human genetic diseases. Traditional gene targeting techniques in embryonic stem (ES) cells, followed by germline chimera production, have been used to generate a vast number of gene-targeted mice [1, 2]. However, this technology is difficult to transfer from mice to large animals, such as swine, sheep and cattle, because the shortage of ES cells derived from domestic animals, and the inherently low efficiency of spontaneous homologous recombination in mammalian somatic cell. It has been demonstrated that the introduction of double-strand breaks (DSBs) in chromosomes can significantly stimulate the efficiency of homologous recombination [3]. Recently, gene knockout can be achieved by using artificial zinc-finger nucleases (ZFNs) to introduce a DSBs at the interest site of chromosomal DNA sequences, which can be be spontaneously repaired by non-homologous-end-joining (NHEJ), in the absence of donor DNA. The NHEJ repair pathway may result in micro-insertions or micro-deletions at targeted region, leading to loss-of-function gene. As a robust gene editing tool, ZFNs successfully modified genomes in various organisms, including *Drosophila* [4], *Arabidopsis thaliana* [5], zebrafish [6, 7], mouse [8], silkworm (*Bombyx mori*) [9], frog [10], rat [11], human [12], pig [13], rabbit [14], cattle [15] and yellow catfish [16], and the targeted modification frequency was up to 50 % without drug selection.

Myostatin (MSTN) is an important negative regulator of muscle growth and belongs to the transforming growth factor-β family [17]. It has been reported that *MSTN* mutation enhanced muscle growth or "double muscle",

Table1 Zinc fingers used in ZFN1 and ZFN2

Finger	Helix	Triplet	Reference number	Source
Left F1 of ZFN1	APSSLRR	GTC	C12-119	CoDA
Left F2 of ZFN1	DSSVLRR	GCC	C12-119	CoDA
Left F3 of ZFN1	QNSHLRR	GGA	C23-116	CoDA
Right F1 of ZFN1	EEVNLRR	GAC	C12-68	CoDA
Right F2 of ZFN1	VRHNLTR	GAT	C12-68	CoDA
Right F3 of ZFN1	DPSNLRR	GAC	C23-68	CoDA
Left F1 of ZFN2	TSTILAR	GTC	C12-254	CoDA
Left F2 of ZFN2	QRSSLVR	GTA	C12-254	CoDA
Left F3 of ZFN2	RTVALNR	GTG	C23-268	CoDA
Right F1 of ZFN2	GGTALVM	AAC	C12-163	CoDA
Right F2 of ZFN2	QSAHLKR	GGA	C12-163	CoDA
Right F3 of ZFN2	DPSNLRR	GAC	C23-176	CoDA

The table is modified from output of http://zifit.partners.org/ZiFiT/CoDAZiFiT/

which happens naturally in cattle, dogs, sheep and humans [18]. Also, it has been demonstrated that homozygous knockout of *MSTN* in mouse by conventional homologous recombination results in increased muscle mass [19], which has prominent economic value for meat production. In livestock, however, *MSTN* knockout has not been achieved. Dong et al. [16] have performed targeted disruption of *MSTN* in yellow catfish using ZFNs; however, no studies reported about the suitability of ZFNs for the targeted modification in the sheep genome. In this study, we used the CoDA (context-dependent assembly) method [20] to design and assemble two pairs of ZFNs, which target against the sheep *MSTN* locus within exon 1 to disrupt the *MSTN* for new sheep breed generation purpose.

Materials and methods

Vector construction

ZFN expression vectors

According to the CoDA method, the ZF binding sites were identified with the tool provided by the web based software (http://www.zincfingers.org/software-tools.htm). Only two potential target sites were screened within MSTN gene exons, and so corresponding two pairs of zinc-finger sequences (Table 1) were synthesized and cloned into pST1374, respectively. To increase the activity of synthesized ZFNs, the wildtype *Fok*I in pST1374 were replaced with Sharkey [21]. Four pairs of ZFN expression vectors, termed ZFN1 (pZFN-1L/pZF-1R), ZFN2 (pZFN-2L/pZFN-2R), ZFS1 (pZFS-1L/pZFS-1R) and ZFS2 (pZFS-2L/pZFS-2R), were constructed.

Table 2 Primers were using in this study

Primer	Sequence
MBS-F	GATCCCGACGGCTCCTTGGAAGACG ATGACTACCACGTTACGACGGAA ACGTTAGC
MBS-R	GGCCGCTAACGTTTCCGTCGTAACG TGGTAGTCATCGTCTTCCAAGGAG CCGTCGG
MSTN-F	ACAGCGAGCAGAAGGAAAATG
MSTN-R	CAGCAGTCAGCAGAGTCGTTG

Reporter vectors

Two pairs of oligonucleotides (MBS-F and MBS-R in Table 2) harboring the *MSTN* ZFN target sequences were synthesized and annealed to cloned into reporter vector pmRFP-eGFP between BamHI/NotI sites, and the obtained reporter vector was named pRGS. In pRGS, the eGFP open reading frame (ORF) was disrupted by an inserted stop codon and a *MSTN* ZFN target site which divided the eGFP ORF into two segments. To facilitate the homologous recombination between the two segments they were set a 200-bp homologous region (supplemental Fig. 1).

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells

HEK293T cells were cultured in Dulbecco's modified eagle's medium (DMEM; Sigma) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin and 10 % (v/v) fetal bovine serum (FBS; Hyclone, Logan, State, USA), at 37 °C in an atmosphere of 5 % CO_2. About 1×10^4 HEK293T cells were seeded into each well of a 24-well plate in 0.5 ml of culture medium and incubated for 24 h before transfection. Culture medium was replaced carefully 1 h prior to transfection and transfection mix was prepared in a 1.5 ml tube as follows: 1 µg DNA in 47 µl Opti-MEM (Sigma) and 2.5 µl Lipofectamine 2000 (Invitrogen) in 47.5 µl Opti-MEM. The solution was mixed carefully, incubated for 25 min at room temperature, and then added dropwise to cells. Cells were then incubated at 37 °C in an atmosphere of 5 % CO_2.

Sheep fetal fibroblasts

Sheep fetal fibroblasts were obtained from an adult sheep as described by Hu et al. [22]. The fibroblasts were maintained in DMEM supplemented with 100 U/ml penicillin, 100 g/ml streptomycin and 15 % (v/v) FBS (Hyclone), at 37 °C in an atmosphere of 5 % CO_2. About 3×10^6 sheep fetal fibroblasts were seeded in 100-mm

dishes in 10 ml of culture medium and incubated for 48 h before transfection. Cells were digested with 0.05 % trypsin–EDTA and resuspended in 10 ml Opti-MEM, then quantified with a hemocytometer. Cells were resuspended in transfection medium [75 % cytosalts (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4; pH 7.6, 5 mM $MgCl_2$) [23], 25 % Opti-MEM] and the cell number was adjusted to 1×10^6 cells/ml. Electroporations were performed in 4-mm gap cuvettes containing 400 µl transfection medium, 1×10^6 cells/ml and 6 µg plasmid with two 1-ms pulses of 500 V, administered through an ECM 2001 electro cell manipulator (BTX, Holliston, MA, USA).

ZFN activity assay

To assay the activity of synthesized ZFNs, HEK293T cells were co-transfected with pRGS and each pair of synthesized ZFNs, respectively, the vector ratio were all 4:3:3. And HEK293 cells transfected with reporter pRGS only were set as control. To test the effect of culture temperature on ZFN activity, the cells were divided into two equal parts and incubated for 3 days at 37 °C, or for 1 day at 37 °C followed by 2 days at 30 °C, respectively. Cells were observed 24, 48 and 72 h after transfection under a fluorescence microscope. Seventy-two hours after transfection, 30,000 cells were counted and the number of RFP express (Rex)-positive cells and eGFP express (GEx)-positive cells evaluated by flow cytometry. The percentage of REx-positive cells represents the efficiency of transfection, and the percentage of GEx-positive cells reflects the activity of ZFNs.

ZFN-targeted MSTN in sheep fetal fibroblasts

Sheep fetal fibroblasts were transfected with the reporter pRGS only, co-transfected with pRGS, pZFN-1L and pZFN-1R (4:3:3), or co-transfected with pRGS, pZFS-1L and pZFS-1R (4:3:3). The transfected cells were incubated for 1 day at 37 °C followed by 2 days at 30 °C. Three days after transfection, the adherent cells were trypsinized and resuspended in DMEM (Sigma) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin and 15 % (v/v) FBS. Single-cell suspensions were sorted using a FACSAria II (BD Biosciences). To collect cells that contain nuclease-induced mutations, cells with strong eGFP signals were sorted. Untransfected cells and cells transfected with reporters alone were used as controls.

T7E1 assay

The T7E1 assay was performed as previously described [8]. Briefly, genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR reactions (50 µl) were carried out using one unit high fidelity Pfu DNA Polymerase (GenScript) in the manufacturer-supplied buffer with 0.4 mM dNTPs and 10 µM primers (MSTN-F and MSTN-R in Table 2). This amplification should produce a 342 bp DNA fragment with the ZFN target site at its center. About 100 ng genomic DNA was used as the template in each PCR reaction. PCR reaction program: denature at 95 °C for 4 min followed by 35 cycles consisting of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; 72 °C for 10 min final extension. The amplicons were denatured by heating and reannealed to form heteroduplex DNA, which was treated with 1 unit of T7 endonuclease 1(T7E1, New England Biolabs) for 1 h at 37 °C and then analyzed by agarose gel electrophoresis.

Analysis of clones derived from single cells

The eGFP-positive FACSAria-sorted cells were plated at a density of 500 cells per 100-mm dish. The culture medium was changed every 3 days. After culture for about 14 days, the cell clones were picked out into 24-well culture dishes to expand the clones. After cells were nearly confluent, they were harvested by trypsin digestion and then divided into two aliquots. One aliquot was expanded to confluence in two wells of a 24-well culture dish and cryopreserved. The other aliquot was used to identify targeting events. Cells were lysed with embryo lysis buffer according to Dai et al [24] and the lysate was used as template for PCR amplification of the targeted locus. The amplified fragment was purified, ligated into pMD18 T and sequenced.

Results

To knock out the *MSTN* gene in sheep, we designed and synthesized two pairs of ZFNs according to the CoDA method. Both of the ZFN-targeted sites were located in exon 1 of *MSTN* (Fig. 1). To determine ZFN activity and to enrich ZFN-targeted cells, we constructed a surrogate reporter in which the ZFN target site cassette disrupts the ORF of an eGFP reporter gene. If the targeted site is cut by the ZFN, a DSB will generated. This will promote homologous recombination between the two repeat regions

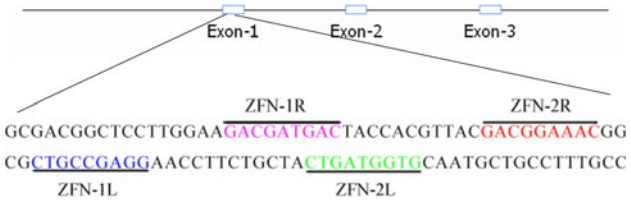

Fig. 1 Schematic diagram indicating the binding of ZFN1 and ZFN2 to the sheep *MSTN* gene

of eGFP gene and restore the expression of eGFP, which can be detected by fluorescence microscopy or flow cytometry [25].

The activity of synthesized ZFNs was determined using the reporter system in HEK293T cells. Seventy-two hours after transfection, a similar number of cells expressing mRFP were detected in all groups of treated cells. Some of the mRFP expressing cells showed simultaneously green fluorescence when transfected with the reporter plasmid and plasmids encoding ZFN1, but few cells expressed eGFP when transfected with the reporter only or when co-transfected with the reporter plasmid and plasmids encoding ZFN2 (data was not shown). These results suggested that ZFN1 can recognize and cut the *MSTN* ZFN target site on pRGS reporter vector more efficiently than ZFN2.

To test the effect of culture temperature on ZFN activity, the cells were divided into 2 equal parts and incubated for 3 days at 37 °C, or for 1 day at 37 °C followed by 2 days at 30 °C, respectively. It was observed that the cells with eGFP expression cultured 1 day at 37 °C followed by another 2 days at 30 °C were a little more than the cells cultured all 3 days at 37 °C but there was no statistically difference (as shown in supplemental Fig. 2).

To enhance the efficiency of the ZFNs activity, we changed the *Fok*I nuclease domain in ZFN1 and ZFN2 with the Sharkey cleavage domain, according to the study of Guo et al. [21]. The resulting ZFN mutants were termed ZFS1 and ZFS2. The activities of ZFS1 and ZFS2 were determined by cotransfection with the reporter system in HEK293T cells. As shown in Fig. 2, flow cytometry indicated that the efficiency of transfection was about 55 % and eGFP was expressed in approximately 15.06 % of cells cotransfected with the reporter plasmid and plasmids encoding ZFN1, in 30.05 % of cells cotransfected with the reporter and ZFS1, in 8.65 % of cells cotransfected with the reporter and ZFS2 and in only 4.14 % of cells transfected with the reporter and ZFN2. These results indicated that the ZFN mutants ZFS1 and ZFS2 can cut the *MSTN* ZFN target site located in the reporter vector with higher efficiency than their wild-type counterparts.

Based on the above findings, we used the ZFS1 plasmids combined with the reporter to disrupt the *MSTN* gene in sheep fetal fibroblasts. The treated cells were analyzed and sorted using a FACSAria II, and approximately 30,000 eGFP-expressing cells from three 100-mm dishes were collected. Only 1.7 % of cells expressed eGFP were sorted (Fig 3). From the eGFP-expressing cells, approximately 10,000 cells were used to isolate genomic DNA for the T7E1 assay, and the remaining cells were plated at a density of 500 cells per 100-mm dish. The T7E1 assay

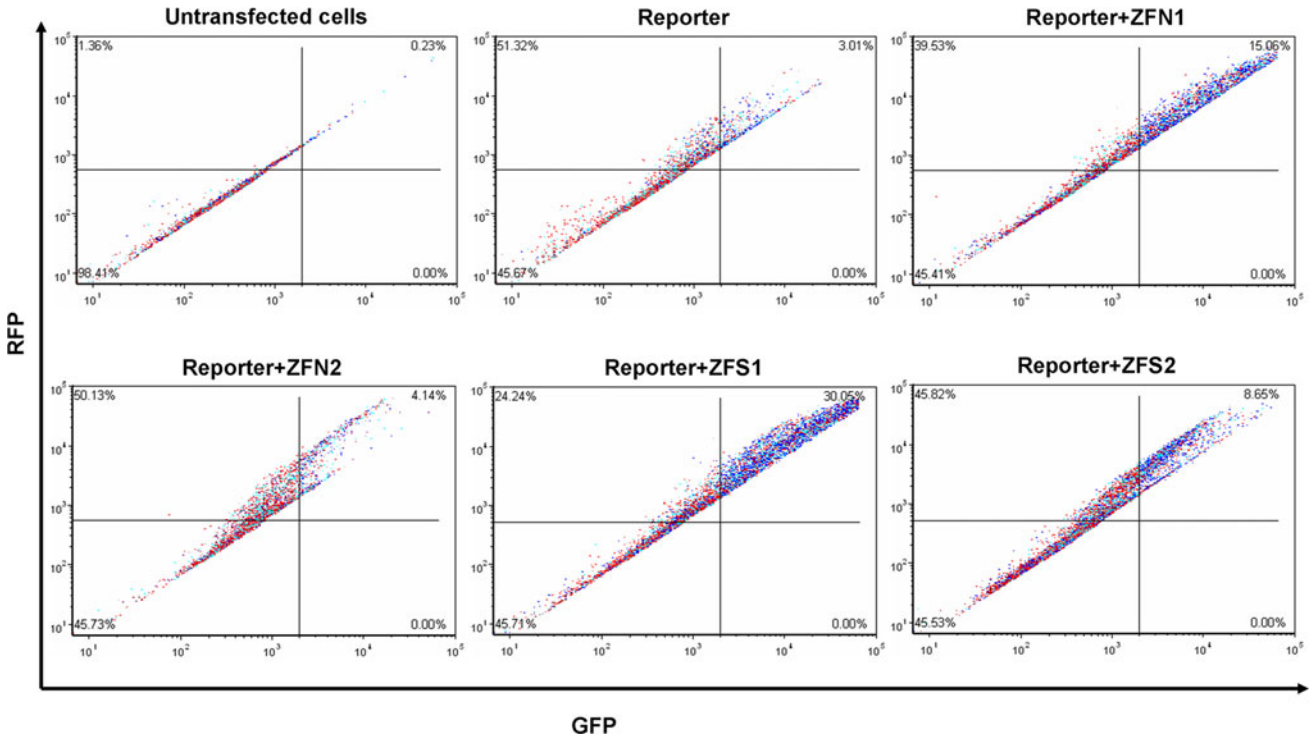

Fig. 2 Efficiency of RFP and eGFP expressions in HEK293T cells cotransfected with a reporter plasmid and plasmids encoding wild-type ZFNs or mutant ZFSs (ZF-Sharkey). HEK293T cells were subjected to flow cytometry 72 h after transfection. Untransfected cells and cells transfected with the surrogate reporter plasmid were used as control and cells transfected with the reporter and plasmids encoding different ZFN paris were analyzed, respectively

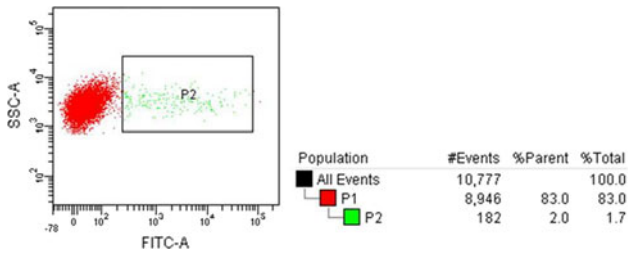

Fig. 3 The surrogate reporter enables efficient enrichment of ZFS1-induced *MSTN*-modified cells assessed with FACSAria II (BD Biosciences). P1 represents non-eGFP-expressing cells; P2 represents eGFP-expressing cells in which *MSTN* gene-modified cells may be enriched

(Fig 4e) displayed three bands but two were very weak in the sorted cells. Only one band was produced from unsorted cells, which suggested that genome-modified cells were enriched by the dual fluorescent reporter vector. After screening 80 clones by sequencing, 2 cell clones with the disruption of *MSTN* were detected. The efficiency of ZFS1 targeting was approximately 2.5 %. As shown in Fig. 4a–c, the sequencing ideograms show double peaks after the targeting site. A 3 bp deletion and a 5 bp deletion in the cutting site were detected by analysis of the sheep *MSTN* gene sequence (no.DQ530260) in the two clones, respectively (Fig. 4d).

Discussion

Gene targeting is a powerful technique for disrupting an endogenous gene, inserting an exogenous gene fragment into a specific locus, or subtly modifying a gene. However, owing to the inherently low efficiency of naturally occurring homologous recombination in mammalian somatic cell, traditional gene targeting cannot be used extensively. It has been reported that the naturally occurring *MSTN* mutant can result in excess muscle development called "double muscle"[26]. *MSTN* has previously been artificially mutated in vitro in bovine and ovine cells, but the efficiency was too low to select a targeted cell clone with expected growth performance [27, 28]. Recently, ZFNs, which can be artificially designed, have shown great potential for targeted modification of specific loci in zebrafish, mouse, rat and even domestic animals such as swine and cattle. With the help of ZFNs, Dong et al. [16] successfully gained some yellow catfish with *MSTN* disruption [16]. However, most of these ZFNs were supplied by Sigma-Aldrich [7] with a extremely high cost, and only a few were synthesized with the modular method [29], OPEN [30], or by the recently developed CoDA method [20]. The main bottlenecks limit the application of ZFNs designed by the OPEN and CoDA methods are relatively impotent specificity or affinity compared with Sigma-Aldrich supplied ZFNs and the lack of a system to enrich or select for targeted cells. And even with the ZFNs supplied by Sigma-Aldrich, some suitable systems may be helpful to enrich targeted cells. By removing Gal^{+} cells with magnetic bead-based method, 99 % of enriched cells were believed to be Gal^{-} analyzed by FACS [13]. The ZFNs synthesized by us using the CoDA method showed low cleavage efficiency, as tested by recovery of eGFP expression and the T7E1 test. T7E1 test was widely used in lots of studies to detect the activity of ZFNs, but it was tedious and not so sensitive.

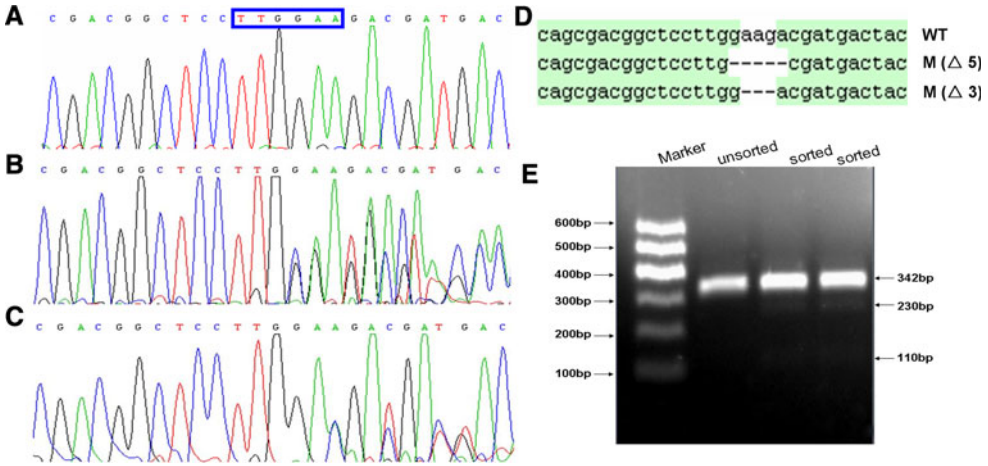

Fig. 4 Representative results of sequencing traces of a wild-type (**a**) and two mutant (**b**, **c**) clones. (**d**) Genetic lesions identified in mutant cells derived from individual clones. The blue box represents the ZFN cutting site; black dashes indicate the positions of deletions in the genomic sequences. (**e**) ZFN-driven mutations detected by the T7E1 assay. The genomic DNA was isolated from the flow-cytometrically sorted cells and unsorted cells, and subjected to the T7E1 assay. The sorted are the MSTN gene-modified cells, which were enriched in the RFP^{+} GFP^{+} cells by flow cytometry as compared to unsorted cells. The bands indicated by the arrows stand for the amplicon cut by T7E1, '*unsorted*' lane has only one band, '*sorted*' lanes have three bands

Firstly, we constructed a surrogate reporter system to detect the activity of ZFNs. The surrogate reporter vector was constructed based on the principle reported by Kim et al. [24]. In our reporter plasmid, instead of being located in the intergenic region between mRFP and eGFP, the target site and a stop codon were inserted in the eGFP ORF, which divided the ORF into two segments. Two direct repeats of 275 bp were introduced on either side of the target site according to Perez-Pinera et al. [31]. Thus, if the target site was recognized and cleaved by the corresponding ZFN, homologous recombination can take place leading to recovery of the eGFP ORF. The expression of eGFP can be detected directly by fluorescence microscope or FACS. Perez et al. [32] reported that when the repeat sequences separated by the target site was 220 bp, up to 50 % of the extra-chromosomal plasmid could be modified, but only 0.5–3.5 % of sites on the chromosome were recovered. Less than 20 % of eGFP loci disrupted by the *MSTN* ZFN target site in pRGS were recovered in our study, which suggested that less chromosomal homologous recombination may occur. Therefore, two methods to enhance the activity of synthesized ZFN and one technique to enrich the targeted cells were employed.

To increase the efficiency of the ZFN, we reduced the temperature of the cell culture according to Doyon et al. [33]. When transfected cells were incubated for 1 day at 37 °C followed by 2 days at 30 °C, there were slightly more cells expressing green fluorescence compared with the other two culture conditions, but these differences were not statistically significant. It has been found that the specificity and affinity of ZFNs can be enhanced by mutation of the *Fok*I nuclease domain. To improve the cleavage efficiency of ZFNs further, the Sharkey domain, described by Guo et al. [25], was chosen to replace the ZFN *Fok*I motif. As reported by Guo et al., Sharkey was 15-fold more efficient than the wild-type *Fok*I domain on a diverse panel of cleavage sites. Our results showed that the cleavage activity of Sharkey was twice than that of the wild-type *Fok*I nuclease.

To select more ZFN targeted cells, the reporter vector used for ZFN activity assay was utilized to enrich the targeted cells. In the surrogate vector designed by Kim [24], which was taken by other researchers in enriching TALEN targeted cells [34], the target site was located between DNA sequences encoding mRFP and eGFP. As described by Kim, only one-third of the reporter plasmids can be fused in-frame to recover eGFP expression owing to the random occurrence of non-homologous end joining. Therefore, many out-of-frame cells may be missed. It has been reported that when DSBs are introduced, the frequency of homologous recombination will increase more than 1,000 times, which is comparable with non-homologous end joining. Liang et al. reported that when a DSB was introduced into one of the two direct repeats, homologous repair can account for 30–50 % of observed repair events, which suggested that homologous recombination is a major DSB repair pathway in mammalian cells [35]. A similar reporter system based on homologous recombination has been used to detect the activity of ZFNs [36]. When reporter plasmids were cotransfected with corresponding ZFNs, the targeted cells were enriched 59 times compared with cells treated with ZFNs only.

In this study, one ZFN induced 3-bp deletion in sheep *MSTN* gene should not alter the open reading frame, just leads to an amino acid delete. The second mutant, a 5-bp deletion, which leads to frameshift mutation, thus it would likely to generate gene inactivation. Furthermore, we will continue to study whether the delete mutation and frameshift mutation make MSTN inactivation to obtain expected growth performance. To date, ZFNs have been successfully used to disrupt α-1, 3-galactosyltransferase (*GGTA1*) and *PPARγ* in pig and β-lactoglobulin *(BLG)* in cattle and they showed great potential for the targeted modification of domestic animals. For the first time, we report the targeted disruption of *MSTN* in sheep using ZFNs. With the help of our surrogate reporter system, a targeted disruption efficiency was up to 2.5 %, which is more efficient compared with traditional gene targeted disruption of *MSTN* in sheep [28]. We propose that our reporter system, together with Sharkey, may facilitate the use of ZFNs in the targeted modification of mammalian genes.

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