



Efficient TALEN-mediated mutagenesis on a highly conserved region of myostatin gene in mouse embryonic stem cells

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ABSTRACT

Myostatin is a negative regulator of muscle growth. The naturally occurring mutations of myostatin gene (*MSTN*) are strongly associated with meat production traits in livestock. Thus far, many attempts have been made to knock-down/knock-out *MSTN* across numerous species. The main objective of this study was to construct and evaluate the efficiency of a single pair of transcription activator like effectors nucleases (TALENs), to target *MSTN* in mouse. A target site in a highly conserved region of *MSTN* exon 2, with minimal off-target counts was selected for targeting by customized TALENs. The targeted site was identified similarly among mouse, cattle, sheep, goat and pig. A modified Golden Gate TALEN generating platform was used for producing the myostatin specific TALEN-expressing plasmids. Generated myostatin TALENs were evaluated in mouse embryonic stem cells (mESC) using high resolution melting (HRM) analysis for detecting newly arising TALEN-induced mutations in the myostatin gene. Our results showed that generated TALENs are functional and able to disrupt the myostatin gene at an efficiency of up to 35% in mESC. If translated to livestock species, this approach can be utilized for producing myostatin modified animals with lower food conversion ratios. The precision and efficiency achieved may even enable direct targeting of zygotes with TALENs, affecting a step-change in the generation of knock-out livestock.

Keywords: Gene targeting, Golden-Gate cloning, High resolution melting, Myostatin, TALENs

Myostatin, also known as growth differentiation factor 8 (GDF8, *MSTN*), is the most effective gene on meat production traits in farm animals. Myostatin has been extensively studied for its clinical and agricultural applications by Rodgers and Garikipati (2008) and Argiles *et al.* (2012), and was found very interesting by livestock breeders and animal scientists due to its major effect on economically important traits (Bellingue *et al.* 2005). The very well known “double muscle” phenotype in the Belgian-

Blue, Piedmontese, and other breeds of cattle (Kambadure *et al.* 1997, McPherron and Lee 1997), along with the Texel sheep (Clop *et al.* 2006) is strongly associated with the naturally occurring mutations in the myostatin gene. Similar evidences for the major effects of myostatin mutations on muscle hypertrophy have also been reported in mouse (McPherron *et al.* 1997), human (Schuelke *et al.* 2004) and dog (Mosher *et al.* 2007). These mutations function as either by disrupting the myostatin gene or by down-regulating the myostatin transcript. These findings support the idea that by applying biotechnological techniques for heritable disruption of myostatin gene in other breeds, more meaty animals can be produced with no severe adverse consequences. Although biotechnology can make huge benefits by generating highly productive local breeds, until recently, livestock transgenesis was limited by the lack of efficient tools for producing genetically modified farm animals. Also there were serious biosafety issues with conventional transgenic techniques which have hindered the required regulations of marketing transgenic products for human consumption. Hence, a genome editing validated tool to be safe and efficient is highly sought after.

The discovery of the simple code of TALEs, transcription activator like effectors (Boch *et al.* 2009, Moscou and

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Bogdanove 2009), and rapidly increasing knowledge of TALE nucleases (TALENs) presented genome-editing researchers with a facile tool to modify the genes in their chromosomal contexts. TALEs are modular DNA binding proteins discovered in the plant pathogen *Xanthomonas* spp. during pathogenesis (Boch *et al.* 2009). The most interesting aspect of these proteins is their repetitive central DNA binding domain which contains a number of highly conserved 33–35 amino acid sequences. These repeats are different in amino acid positions 12 and 13 (RVDs: repeat variable di-residues). Based on the RVDs, there is a one-to-one correspondence between a repeat in DNA binding domain of TALE and a base on the targeted sequence of DNA (Cermak *et al.* 2011). By engineering the central DNA binding of TALEs, new proteins with the ability of recognizing and binding a specific sequence throughout the genome can be produced (Bogdanove and Voytas 2011). TALENs are DNA cutting proteins, which are artificially generated by replacing the native activation domain of TALEs with a non-specific nuclease domain, usually of *FokI*. As *FokI* nuclease domain needs to be dimerized for cutting the DNA, a pair of TALENs can be easily customized to introduce a double strand break (DSB) at the targeted locus (Miller *et al.* 2011). TALEN-induced DNA lesions would be repaired by one of the DNA repair machinery pathways; the dominant but error prone non-homologous end joining (NHEJ) and the less frequent but accurate homology-directed repair (HDR). Therefore, it is highly possible to mutate a TALEN-targeted gene by insertion/deletions occurred during NHEJ repair.

Myostatin signaling has been blocked by use of conventional homologous recombination (HR) techniques (Zhang *et al.* 2007, Zheng *et al.* 2012), single strand DNA oligonucleotides (Grisolia *et al.* 2009), injection of blocking myostatin antibodies (Bogdanovich *et al.* 2002) and short hairpin RNA interference technology (Tripathi *et al.* 2013). However, the recently developed genome-editing tool, TALENs, represent a more efficient way for myostatin gene targeting (Carlson *et al.* 2012, Tan *et al.* 2013, Xu *et al.* 2013). The main aim of this study was to generate and evaluate a pair of TALENs for targeting myostatin gene in mouse embryonic stem cells (mESC) which could be applied in cattle, sheep, goat, pig and mouse. Herein, we used the Golden-Gate cloning method with modifications to generate TALENs targeting a conserved region in the second exon of the myostatin gene, and compared the efficiency of these in-house generated TALENs with commercially obtained TALENs for targeting myostatin gene.

MATERIALS AND METHODS

All plasmid DNA extractions were performed using a commercial kit. Sequencing was performed at the Gandel Charitable Trust Sequencing Centre facilities, Monash University, Australia. Primers were obtained commercially and listed in the Supplementary Table 1.

Target site selection: The sequences corresponding to

the myostatin coding regions of mouse, cattle, sheep, goat and pig were downloaded from the NCBI and aligned using CLC Main Workbench software 6. Highly conserved regions of myostatin gene were analyzed using TALENTM Hit software (<http://talen-hit.collectis-bioresearch.com/search>). BLAST analysis was performed against mouse, cattle, goat, sheep and pig reference genomes to determine the uniqueness of the predicted target sites. Off-target counts for each species were predicted by using TALEN-NT 2.0 software (Doyle *et al.* 2012). Ultimately, a region (TN₁₆-Spaser₁₆-N₁₆A) located in exon 2 which was found to be identical in all mentioned species with the least off-target counts was selected for targeting (Supplementary Fig. 1).

TALENs construction: Golden Gate cloning kit (Cermak *et al.* 2011) and 2 TALEN backbone vectors, pCS2TAL3DD and pCS2TAL3RR (Addgene plasmids 37275 and 37276, respectively) (Dahlem *et al.* 2012) were procured commercially for TALEN construction. Three new backbone plasmids pCS2TAL3DD-IRES-EGFP, pCS2TAL3RR-IRES-mCherry and pCS2TAL3-RR-SpnR were generated from pCS2TAL3DD and pCS2TAL3RR (Supplementary Figs. 2–4).

A pair of TALENs was constructed using a modified Golden Gate cloning protocol (Cermak *et al.* 2011), where, 2 rounds of Golden Gate reactions used for cloning the left TALE sequence into pCS2TAL3DD-IRES-EGFP backbone and the right TALE sequence into either pCS2TAL3-RR-IRES-mCherry or pCS2TAL3-RR-SpnR backbones (Fig. 2). Correctly assembled plasmids were confirmed by sequencing. In addition, a third round of digestion/ligation reaction was added to the protocol which was used for recombining MSTN-pCS2TAL3DD-IRES-EGFP and MSTN-pCS2TAL3RR-SpnR to clone both left and right TALENs in one single plasmid, MSTN-GGIII (Fig. 2). This reaction consisted of 75 ng of each MSTN-pCS2TAL3DD-IRES-EGFP and MSTN-pCS2TAL3RR-SpnR plasmids, 10 units of each *Sall*-HF, *XhoI*, *XbaI*, *NheI*-HF, T7 DNA ligase enzymes in 1×NEB smart buffer supplemented by 10 mM ATP. The reaction was incubated for 10 cycles including 5 min digestion at 37°C and 5 min ligation at 25°C following by addition of 10 units exonucleaseV (RecBCD) and incubation at 37°C for 30 min. The products were directly transformed into *E. coli* DH5α competent cells and plated on ampicillin and spectinomycin antibiotics screening plates. Recombinant plasmids were extracted and confirmed by digestion.

In addition to the in-house generated TALENs, a pair of TALEN-encoding plasmids was procured commercially for targeting the same region. The functionality of the Collectis-MSTN-TALENs was confirmed by the company, using single strand annealing (SSA) assay in yeast (Supplementary Fig. 5 for plasmid maps and the SSA assay principle).

In-vitro validation of TALENs in mESC: Mouse ESC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 µg/ml penicillin and streptomycin, 1% non-essential amino acids, 10% fetal

bovine serum (FBS), 1000 U/ml of ESGRO LIF and 0.1 mM beta-mercaptoethanol. Mouse ESCs were treated with Tryple at 37°C to obtain a single cell suspension. After neutralization with the medium, 4×10^5 mESC were transferred into each well of a gelatin coated 6-well plate and at the same time were transfected using the lipofectamine LTX with Plus™ Reagent according to the manufacture's instruction. Three separate transfections were conducted including, first: co-transfection of 2 µg of each MSTN-pCS2TAL3DD-IRES-EGFP and MSTN-pCS2TAL3RR-mCherry plasmids; second: 2 µg of each left and right Collectis-MSTN-TALENs and the third: transfection of 4 micrograms MSTN-GGIII plasmid. Cells were incubated at 37°C with 5% CO₂ and transfection efficiency checked by fluorescent microscopy 12 h after transfection. Three days after transfection, single colonies were picked up and expanded into gelatin coated 96-well plates.

Mutation analysis: Colony selection and mutation screening were carried out. Briefly, 96-well plates containing isolated mESC colonies were passaged into 2 separate plates, with 1 of them frozen as a back-up plate and the other used for HRM analysis. To extract DNA from colonies in 96-well plates, the medium was aspirated and cells were washed with PBS followed by treatment of 30 µl Tryple for 10 min at 37°C. Single cell suspensions were transferred into microtubes and heated at 95°C for 10 min in a thermocycler. One microliter of the resulting heated cell lysate was used as the DNA template in HRM reactions. Two combinations of primers were tested in HRM analysis including Myo97F/MyoHRMR and Myo336F/MyoHRMR which amplify 97 bp and 336 bp regions bordering the TALEN target site, respectively (Supplementary Table 1). Amplifications were carried out in 10 µl PCR reactions containing: 1× HRM MasterMix, 2.5 µM each forward and reverse primers and 1 µL DNA. The thermal and cycling conditions were: 1 cycle denaturation at 95°C for 2 min, 45 cycle amplifications including 94°C for 30 sec and 67°C for 30 sec, 1 cycle heteroduplex formation 94°C for 30 sec and ramping down (0.1°C per sec) to 25°C. HRM data collection performed on a Light Scanner and analyzed using the LightScanner Call-IT 2.0 Software.

For confirming the mutants detected by HRM analysis, 3 mESC colonies were further analyzed by sequencing. For that, MyoT7EIF/MyoT7EIR primers were used to amplify a 904 bp region flanking myostatin exon 2 using Platinum Taq DNA polymerase high fidelity according to the manufacture's instruction. The amplified fragments, were then cloned into pGEMt-Easy vector and 5 *E. coli* colonies were sequenced for each of mESC colonies. Sequencing results were analyzed for the detection of TALEN-induced mutations.

RESULTS AND DISCUSSION

Bioinformatics tools were successfully used for finding a highly conserved region of myostatin gene, which was identical in different species. Based on the unique TALEN

target site in the genome MSTN-TALENs were generated (Supplementary Fig. 1).

The Golden Gate TALEN generating platform (Cermak *et al.* 2011) was used for generating MSTN-TALENs with modifications. The Golden Gate cloning system provides a cost and time efficient approach for constructing TALEN-encoding plasmids. In our hands, nearly all of the constructed TALENs were correctly assembled. Although the system was originally designed for use in yeast (Cermak *et al.* 2011), further enhancements were made to construct vectors suitable for mammalian cell applications (Bedell *et al.* 2012, Carlson *et al.* 2012, Frank *et al.* 2013, Perez-Pinera *et al.* 2013, Sakuma *et al.* 2013). In this study, we first added 2 fluorescent proteins GFP and mCherry sequences into the backbone plasmids pCS2TAL3DD and pCS2TAL3RR, respectively. These modifications enabled us to determine the transfection efficiencies, as well as to identify under a fluorescent microscope those colonies that had received both left and right TALENs. We also generated a plasmid containing left and right TALEN coding sequences. Having both left and right TALENs in a single plasmid, made us confident that both are expressed in transfected cells. However, our fluorescent microscopy observations indicated that transfection efficiency and having both TALENs on a single plasmid may not be critical factors at least in highly transfectable cells such as mESC. In our hands, all of the mouse ES colonies that have been co-transfected with the left and right TALENs in separate plasmids received either both or none of the plasmids. This may be due to the high efficiency of transfection in mESC, and different results may be obtained with hard-to-transfect cell lines (Feng *et al.* 2014). In addition to the 2 rounds of reactions in Golden Gate cloning system, a third digestion/ligation reaction can be easily setup using the backbones modified in this study to efficiently put both left and right TALENs in one plasmid. This reaction took advantages of restriction enzymes, which can work in the same buffer and produce fragments with compatible ends. In the presence of a ligase, digestions and ligations can take place in a single micro-tube during cycles. Therefore, recombinant plasmids can be easily constructed without the laborious procedures of conventional genetic engineering techniques. Although the method presented in this study focus on MSTN-TALENs, we have successfully used the same for assembling other TALENs in one plasmid.

The ability of generated MSTN-TALENs for inducing mutations at myostatin locus was tested in mESC. Mouse ESC were selected for evaluating the MSTN-TALENs because of their availability, ease of handling, and their colony-formation properties, which enabled pick-up and expansion of the transfected cells. The fluorescent signal of EGFP and mCherry proteins, co-expressed with the MSTN-TALENs in mESC, were easily detectable 3 days after transfection which enabled us to isolate colonies that have received both left and right TALEN encoding plasmids (Supplementary Fig. 6), thereby enriching for targeting outcomes. Although the high efficiency of TALENs allows

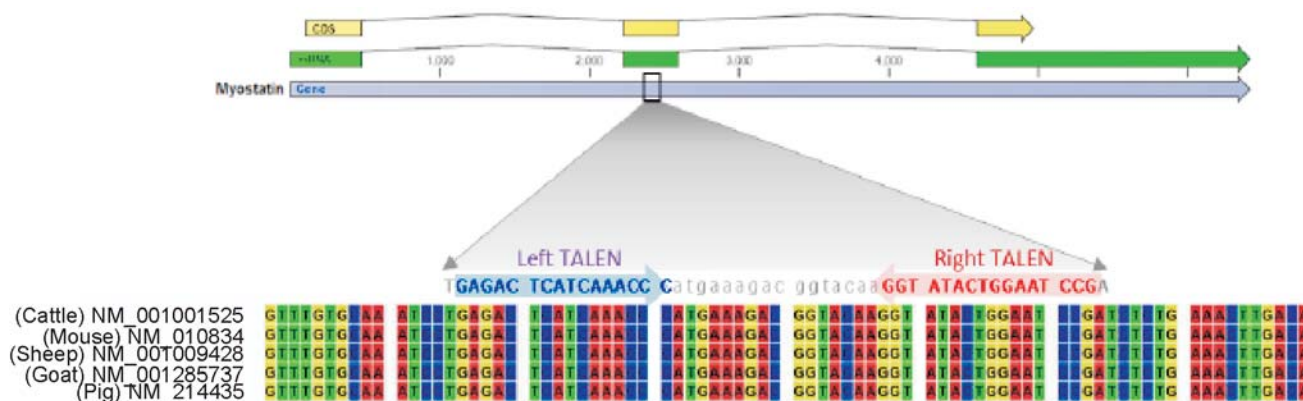


Fig. 1. Alignment of myostatin genomic sequences flanking TALEN target sites in mouse, cattle, sheep, goat and pig.

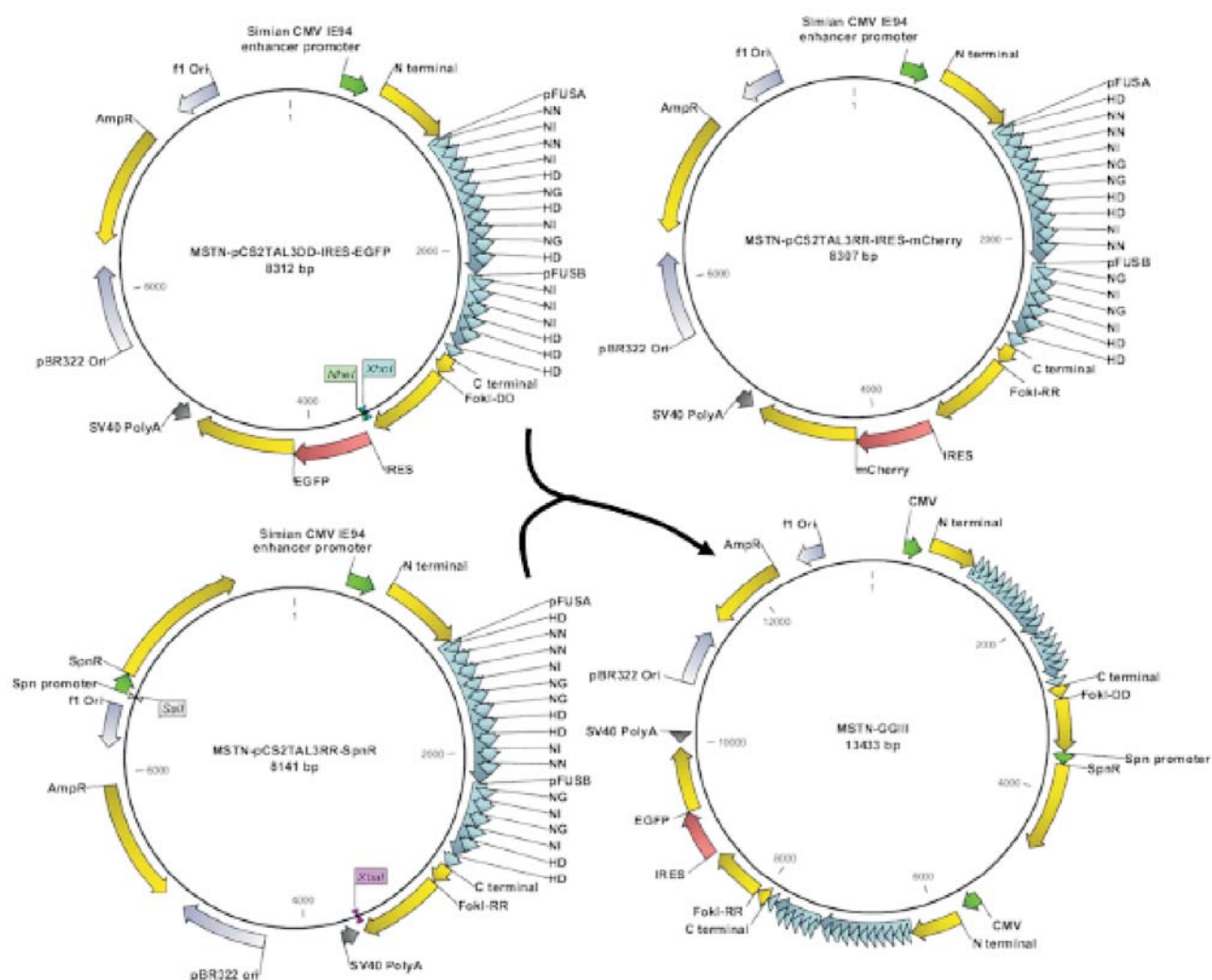


Fig. 2. MSTN-TALEN expressing plasmids constructed and evaluated in this study. EGFP, enhanced green fluorescent protein, IRES, internal ribosome entry site.

researchers to establish knock-out cell lines without aid of antibiotic selection markers, nevertheless, being able to specifically pickup transfected cells by fluorescence for downstream genotyping steps would even lead to achieving higher efficiencies, which are especially relevant if coupled

with direct delivery of TALENs to zygotes. It has been demonstrated that enrichment of cells (Feng *et al.* 2014) and embryos (Carlson *et al.* 2012) by transfection of TALEN plasmids containing fluorescent proteins greatly increases the proportion of mutated cells/embryos. Similar

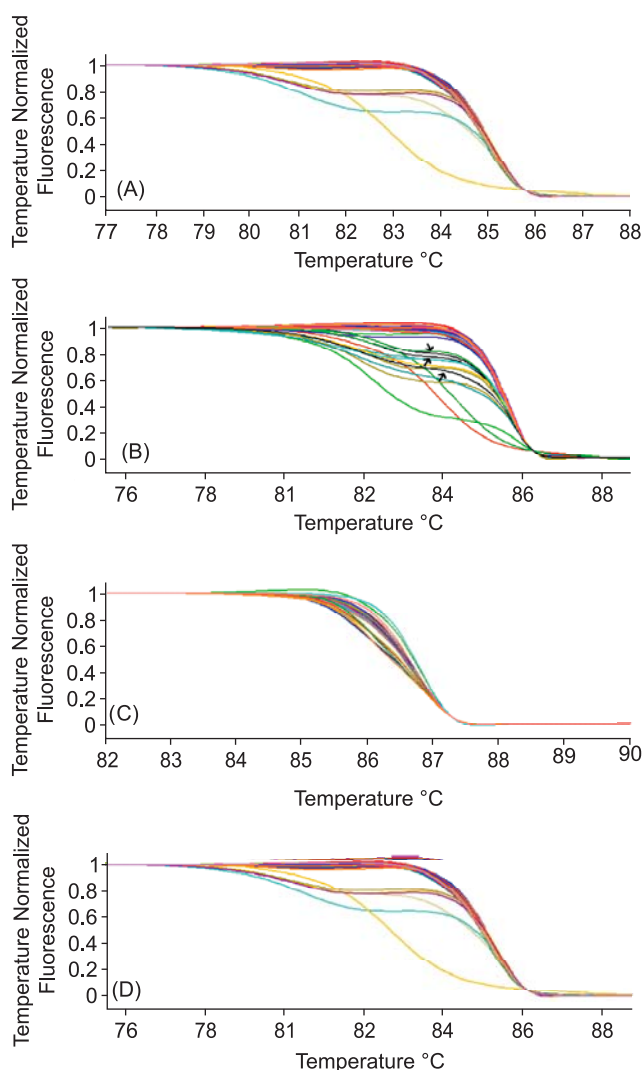


Fig. 3. TALENs-mediated mutations on mouse myostatin locus detected by high resolution melting (HRM) analysis. (A) HRMA results for mESC transfected with Collectis-MSTN-TALENs. (B, and C) HRMA results for mESC transfected with pCS2TAL3DD-IRES-EGFP and pCS2TAL3RR-IRES-mCherry using 97 bp and 336 bp amplifying primers, respectively. (D) HRMA results for mESC transfected with MSTN-GGIII.

results were obtained when TALENs are co-transfected along with an antibiotic resistant plasmid (Wang *et al.* 2013, Xin *et al.* 2013).

HRM analysis methodology was used for detecting TALEN-mediated mutations in mESC colonies. HRM assay successfully detected mutant mESC (Fig. 3). Shorter DNA fragment, 97 bp, produced more distinguishable melting curves comparing to the 336bp fragment (Fig. 3B-C). Thus far, different approaches including *Cell/T7* endonuclease I assay, and restriction fragment length polymorphism (RFLP) were used for detecting TALEN-induced mutations. However, HRM analysis provides a rapid, simple and high throughput method to accelerate detection of unknown mutations generated by TALENs (Dahlem *et al.* 2012, Panda *et al.* 2013). Due to the amplification of a short fragment flanking the TALEN target site, HRM assay is not able to detect mutations that disrupt the primer binding sites. In this study, HRM analysis detected 6, 14 and 6 mutants out of 70, 40 and 22 isolated mESC colonies, respectively, (co-) transfected by the pair of the Collectis-MSTN-TALENs, pair of the MSTN-pCS2TAL3DD-IRES-EGFP and MSTN-pCS2TAL3RR-IRES-mCherry and finally the MSTN-GGIII single plasmid. Although all constructs encoded TALENs that target the same sequence on myostatin gene, however, a main reason that more mutants have been detected in mESC (co-)transfected with TALENs-fluorescent protein fusion plasmids is due to being able to carry out the analysis only on fluorescent positive cells.

Generated TALENs in this study took the advantages of obligate heterodimer *FokI* nuclease, which make them more specific. The efficiency achieved in this study, up to 35%, is comparable with targeting rates (ranging up to 60%) reported in other studies targeted genes in different mammalian cell types by using TALENs (Supplementary Table 2). The efficiency of TALENs was reported to be even higher (up to 77%; Supplementary Table 2) when directly injected into the one-cell stage embryos. The possibility of obtaining one-step generated knock-outs, by embryo-based TALEN gene targeting, may have tremendous impact on the production of genome-edited livestock species, where pluripotent cells with germ-line

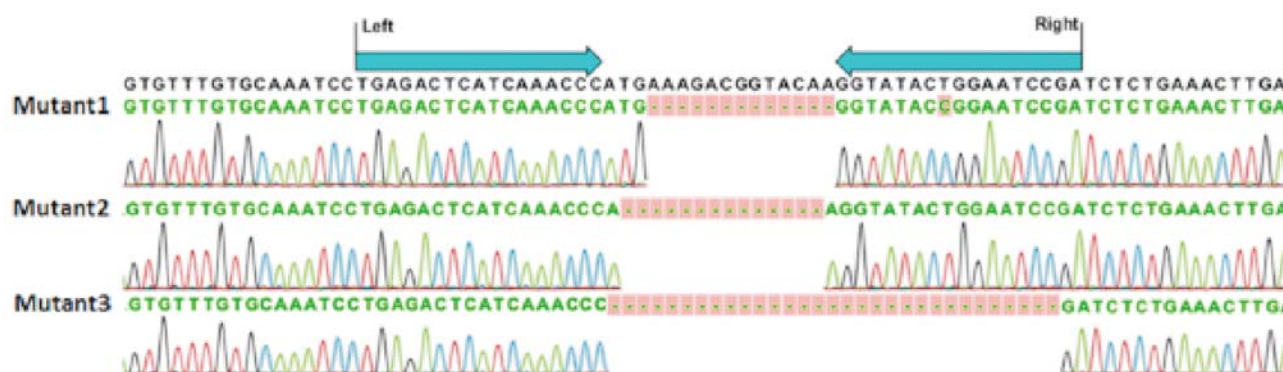


Fig. 4. Sequence analysis of mutant mouse ES cell colonies. Their corresponded melting curves in HRM analysis are indicated in Fig. 3B.

transmission ability are not yet available.

To validate the HRM analysis results, 3 detected mESC colonies were selected for confirmation of mutation by sequencing (Fig. 4). All 3 NHEJ events in myostatin locus sequenced in this study were deletions that cause frame shifts. It has been demonstrated that TALENs intend to introduced deletions rather than insertions in their target sites (Kim et al. 2013).

Obviously, TALENs efficiency for targeting myostatin genes (Carlson et al. 2012, Tan et al. 2013, Xu et al. 2013) is greatly higher than efficiencies obtained by conventional HR techniques (Zhang et al. 2007, Zheng et al. 2012). In addition to the higher efficiency, TALENs have more advantages over the conventional HR technique, making them superior means for establishing gene-targeted cell lines or organisms. Notably, TALENs can be used as mRNA and directly injected into the single-cell stage embryos, without a need for antibiotic selection. Using TALENs as mRNA reduces the risk of random integration of the foreign DNA into the genome as well as avoiding unknown mutagenic effects.

In conclusion, here we evaluated the efficiency of a pair of TALENs targeting a highly conserved region of myostatin gene. Although in this study, mESC were selected for evaluating MSTN-TALENs, however, we speculated that these pair of TALENs could be used for targeting myostatin gene in livestock as well. Also, we reported here the construction of new backbones encoding fluorescent proteins using Golden Gate cloning system, which allowed us to ensure suitable expression of TALENs as well as generating a single plasmid encoding both left and right TALENs.

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