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Myostatin knockout using zinc-finger nucleases promotes proliferation of ovine primary satellite cells in vitro

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ABSTRACT

Myostatin (MSTN) has previously been shown to negatively regulate the proliferation and differentiation of skeletal muscle cells. Satellite cells are quiescent muscle stem cells that promote muscle growth and repair. Because the mechanism of MSTN in the biology of satellite cells is not well understood, this study was conducted to generate MSTN mono-allelic knockout satellite cells using the zinc-finger nuclease mRNA (MSTN-KO ZFN mRNA) and also to investigate the effect of this disruption on the proliferation and differentiation of sheep primary satellite cells (PSCs). Nineteen biallelic and four mono-allelic knockout cell clones were obtained after sequence analysis. The homologous mono-allelic knockout cells with 5-bp deletion were used to further evaluations. The results demonstrated that mono-allelic knockout of MSTN gene leads to translation inhibition. Real-time quantitative PCR results indicated that knockout of MSTN contributed to an increase in CDK2 and follistatin and a decrease in p21 at the transcript level in proliferation conditions. Moreover, MSTN knockout significantly increased the proliferation of mutant clones ($P < 0.01$). Consistent with the observed increase in CDK2 and decrease in p21 in cells lacking MSTN, cell cycle analysis showed that MSTN negatively regulated the G1 to S progression. In addition, knockout of myostatin resulted in a remarkable increase in MyoD and MyoG expression under differentiating conditions but had no effect on Myf5 expression. These results expanded our understanding of the regulation mechanism of MSTN. Furthermore, the MSTN-KO ZFN mRNA system in PSCs could be used to generate transgenic sheep in the future.

1. Introduction

Satellite cells represent a unique population of muscle precursor cells that lie between the basal lamina and sarcolemma of the muscle fiber and are the myogenic stem cells of vertebrate skeletal muscle (Mauro, 1961). These cells are a population of mononuclear cells that have strong proliferation and differentiation capacities (Wu et al., 2012). Satellite cells are considered mitotically quiescent until stimulated by growth factors to proliferate, differentiate, and fuse to existing muscle fibers (Dodson et al., 1996). Among the most studied growth factors that have specific effects on proliferation and differentiation is the transforming growth factor-beta (TGF-β) family. They are actively involved in the control of proliferation and differentiation of several myogenic cell lines. Myostatin (MSTN) is a secreted growth and differentiation factor (GDF-8) that belongs to the TGF-β family. Myostatin expression is found during embryonic and postnatal stages of growth; thus, myostatin may play a key regulatory role throughout myogenesis (Kambadur et al., 1997; McPherron et al., 1997). Several studies have investigated the role of MSTN in satellite cells. For example, the effects of endogenous myostatin on turkey embryonic satellite cells (McFarland et al.,

Abbreviations: PSC, primary satellite cell; TGF-β, transforming growth factor β; CDK2, cyclin-dependent kinase 2; p21, cyclin-dependent kinase inhibitor 1; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; RIPA, radio immunoprecipitation assay; HRP, horseradish peroxidase.

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1993) and mice satellite cells (McCroskery et al., 2003) have been reported, whereas no studies have reported on the role of MSTN in *ovine* primary satellite cells (PSCs).

Previous studies in sheep have focused on using knockout methods to inactivate the myostatin gene. Recently, zinc-finger nuclease (ZFN) technology has provided powerful tools for genome engineering that enables targeted mutagenesis. As a robust gene editing tool, ZFNs have been used to successfully modify genomes in various organisms, including insects, amphibians, plants, and mammals. This broad range of tractable species renders ZFNs a useful tool for improving our understanding of complex physiological systems and for producing transgenic animals. ZFNs, which can be artificially designed, have shown great potential for creating targeted modifications of specific loci in zebrafish (Meng et al., 2008), mouse (Meyer et al., 2010), rat (Chu et al., 2012), yellow catfish (Dong et al., 2011) and even domestic animals such as swine (Hauschild et al., 2011) and cattle (Yu et al., 2011). With the help of ZFNs, Zhang et al. (2014) successfully produced sheep fetal fibroblasts with a MSTN disruption at the exon 1 site; however, no studies have reported on the suitability of ZFNs for targeted modification of the genome of sheep satellite cells.

In this manuscript, we have shown that MSTN-KO ZFN mRNA introduces a 5-bp deletion at the exon 3 site of the MSTN gene in sheep, which leads to the translational inhibition of the MSTN gene and thus increases *ovine* PSCs proliferation. In addition, we have used the mono-allelic MSTN knockout satellite cell clones as an *in vitro* model for studying the role of MSTN in satellite cell proliferation, differentiation and cell cycle progression. Finally, real-time quantitative PCR (RT-PCR) analysis was carried out to detect the transcript levels of FST, p21, CDK2, MyoD, Myf5 and MyoG, which are genes that play a role alongside MSTN in regulating muscle mass.

2. Materials and methods

2.1. Zinc-finger nuclease design and donor vector construction

MSTN-KO ZFNs mRNA were designed to target the exon 3 site of myostatin gene in sheep muscle PSCs by binding to a user-specified locus and causing a double-strand break. MSTN-KO ZFNs mRNA was purchased from Sigma-Aldrich. Detailed sequence information about the zinc finger modules can be found in Fig. 1A.

Donor plasmids were created to correspond to the cleavage location of the two ZFN pairs. Two pairs of homologous arms (RA and LA) harboring the MSTN ZFN target sequences were synthesized and annealed to be cloned into vector pEGFP-N1. The primers used in this study are listed in Table 1. A homologous RA (446 bp) and homologous LA (452 bp) were located in the intron 2 area and exon 3 area of sheep MSTN gene, respectively. Finally, LA was inserted into the Not1/Afl II site and RA was inserted into the ApaLI/Afl III site of the pEGFP-N1 vector. The donor vector thus obtained was named pGNM (Fig. 1B).

Table 1
Primers used for sheep MSTN gene amplification.

Primers	Primer sequences	5 Restriction site	Length of product (bp)
LAF	GGAGAGATTTTGGGCTTGATTGTG	Not I	446 bp
LAR	TGACTGTAGCATACTCTAGGCTT	Afl II	
RAF	AAGTGAATGGAGAATGAATGAGT	ApaL I	452 bp
RAR	AGGAAAGAAGAGTTAGTAATAGA	Afl III	

RAF and RAR were used for amplifying MSTN gene homologous right arms from sheep fetal Satellite Cell genomic DNA and LAF and LAR were used for amplifying MSTN gene homologous left arms from sheep fetal Satellite Cell genomic DNA.

2.2. Preparation, culture and transfection of primary satellite cells

Sheep PSCs were isolated from female fetuses at day 50 of gestation as previously described (Doumit and Merkel, 1992). Isolated satellite cells were seeded on 2% matrigel (BD Biosciences, Bedford, MA) coated 6-well plates and cultured in growth medium (GM) containing DMEM supplemented with 20% fetal bovine serum and antibiotics at 37 °C and 5% carbon dioxide (CO_2). Identification of sheep PSCs was performed by immune-fluorescence as described by Wu et al. (2012).

Cellular delivery was performed using lipid-based transfection reagents (TransIT-mRNA Transfection Kit, Mirus Company). The cells were co-transfected with ZFNs and GFP donor plasmids as recommended by the manufacturer for first and second time transfection. Briefly, the cells were seeded in 2 × 6-well plates and cultured until 80% confluence. The day before transfection, we removed the medium and added 2 ml of fresh medium to the cells prior to the transfection. The experimental mixture was prepared by adding the following reagents per sample: 5 μl of donor plasmid, 5 μl of ZFN mRAN, 100 μl of serum-free medium, 2.5 μl of TransIT mRNA reagent, and 1.25 μl of MRNA boost reagent. The mixture was incubated at room temperature for 2 min and then added to the cells dropwise. Next, the cells were incubated in a CO_2 incubator at 37 °C for 24 h and 48 h (days 1 and 2), and culturing was continued in fresh GM for 3 days following the first transfection (day 5). One well of the each plates were then randomly selected and digested, genomic DNA was purified, and PCR amplification of CEL-I was performed. After a positive CEL-I assay, a second co-transfection was performed as above using 5 wells of a plate for 48 h (day 7) and the remaining well plates were collected for further analysis. Three days after the second transfection (day 10), the treated cells were tested for CEL-I. After positive CEL-I results, the cultured cells were single cell cloned.

2.3. DNA cleavage assay

The frequency of targeted gene mutation in ZFN-treated cells was determined using the CEL-I nuclease assay. The sequences of PCR primers used for amplification of the MSTN target locus were as follows: forward primer 5′-GAGCCATAAAGGCAGAATCAAGC-3′ and reverse primer 5′-GTTAGAGGGTAACGACAGCATCG-3′. A 285-bp PCR product was generated from a wild-type genomic template. A CEL-I assay was performed on genomic DNA extracted from control cells, cells transfected with ZFN only (ZO), cells transfected with donor vector only (DO), and cells derived from the first and second transfection with donor vector and ZFN (DZT1 and DZT2, respectively). The DNA from the control, ZO, DO and DZT1 cell groups was isolated on day five of transfection, whereas for DZT2 cells, the DNA was isolated on day ten of transfection.

2.4. FACS analysis of GFP expression and single-cell sorting

GFP expression was used for fast detection of integration-positive clones in subsequent FACS analysis. GFP expression in PS cells was photographed under a fluorescent microscope at days 1, 2 and 7 of transfection. Analysis of GFP expression was also performed using the FACS (MoFlo® Astrios™) system. For FACS analysis of GFP expression, the cells were collected at days 1, 2, 5 and 7 of transfection by centrifugation and washed in PBS.

GFP-positive single-cell sorting from live population cells of the second transfected pool was performed using a FACS (MoFlo®Astrios™) system in 7 × 96-well plates.