

Production of MSTN mutated cattle using CRISPR–Cas9

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Abstract

Many transgenic animals have been produced using CRISPR–Cas9 technology to edit specific genes. However, there are few guidelines for the application of this technique in cattle. The goal of this study was to produce trait-improved cattle using the genome editing technology CRISPR–Cas9. Myostatin (MSTN) was selected as a target locus and synthetic mRNA of sgRNA and Cas9 was microinjected into bovine in vitro fertilized embryos. As a result, 17 healthy calves were born and 3 of these showed MSTN mutation rates of 10.5%, 45.4%, and 99.9%, respectively. Importantly, the offspring with the 99.9% MSTN mutation rate had biallelic mutation (-12bp) and a doubling muscle growth phenotype. In conclusion, we showed that the genome editing technology CRISPR–Cas9 can produce genetically modified calves with improved traits.

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Abbreviations: **MSTN**, myostatin; **sgRNA**, single guide RNA; **ART**, advanced reproductive technologies; **SCNT**, somatic cell nuclear transfer; **T7E1**, t7 endonuclease 1

Abstract

Many transgenic animals have been produced using CRISPR–Cas9 technology to edit specific genes. However, there are few guidelines for the application of this technique in cattle. The goal of this study was to

produce trait-improved cattle using the genome editing technology CRISPR–Cas9. Myostatin (*MSTN*) was selected as a target locus and synthetic mRNA of sgRNA and Cas9 was microinjected into bovine in vitro fertilized embryos. As a result, 17 healthy calves were born and 3 of these showed *MSTN* mutation rates of 10.5%, 45.4%, and 99.9%, respectively. Importantly, the offspring with the 99.9% *MSTN* mutation rate had biallelic mutation (-12bp) and a doubling muscle growth phenotype. In conclusion, we showed that the genome editing technology CRISPR–Cas9 can produce genetically modified calves with improved traits.

Many animal product (milk and meat) studies focus on the improvement of performance traits in cattle because cattle contribute 45% of the global animal protein supply for human consumption [1,2]. Significant effort has been made to improve the trait of cattle using advanced reproductive technologies (ART) [3,4] based on genotyping and phenotyping analysis. One application of genotyping and phenotyping breeding is to select and propagate the breeds with high amount of muscle. Belgian Blue and Piedmontese are the most representative double muscles cattle breeds. [5]. Genetic analysis identified the mutation of *MSTN* (growth and differentiation factor 8) as the causative factor for enhanced muscle development. Mutations in the gene have also been observed in the dog [6], sheep [7], pig [8] and human [9]. However, the incidence of these natural mutations is very low and selecting and breeding these individuals to establish an independent breed is time-consuming and costly.

The development of genome editing tools such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR–Cas9) has provided new and powerful gene editing tools for functional mutation studies in various biotechnological industries. Applying these genome editing tools to livestock will contribute to: improvements in cattle traits, and better understanding of how to prevent and treat cattle disease [10]. Proof of concept gene edited cattle—using bovine somatic cell nuclear transfer (SCNT)—have been produced with enhanced traits for disease resistance, allergen removal, production, and welfare [11–15]. Because SCNT is used to produce cloned offspring with precise gene edited cells, it is employed in cattle to generate a valuable model [16,17]. However, abnormal reprogramming results in low efficiency and survival rate, limiting the application of SCNT. An alternative approach to increase the efficiency of gene editing is through Belgian Blue the microinjection of in vitro fertilized embryos [18]. One study used TALENs for editing *MSTN* gene microinjection of in vitro fertilized cattle embryos [19]. However, there are no reports of a live-born genome edited calf using CRISPR–Cas9 on the *MSTN* locus. Accordingly, the aim of present study is 1) to develop a method to produce gene edited bovine pre-implantation embryos using through microinjection with Cas9 mRNA and 2) to produce the *MSTN* edited calves.

Materials and methods

Single guide RNA design and testing on bovine fibroblasts

To produce the *MSTN* mutated cattle, single guide RNA (sgRNA) for *MSTN* targeting exon2 was designed by CHOPCHOP software (<https://chopchop.cbu.uib.no/>) that selects sgRNA candidates for target genome (Fig 1A-a). Then the sgRNA for *MSTN*, and Cas9 protein were co-transfected on bovine fibroblast cells. After 3 days of transfection, they were harvested for extraction of genome DNA. Through the T7E1 assay, it was confirmed whether the target *MSTN* gene was mutated.

Oocyte in vitro maturation (IVM)

Ovaries were obtained from a local slaughterhouse and delivered to the laboratory within 2 hours. The ovaries were aspirated with a 18-gauge needle to obtain cumulus-oocyte complexes (COCs) from follicles with 2–8 mm in diameter. The COCs with more than three layers of cumulus cells and evenly distributed cytoplasm were sorted in the study. For IVM, the COCs were cultured in chemically defined TCM-199 supplemented with 0.005 IU/mL FSH (Sigma–Aldrich, Cat. no. F2293), 1 µg/mL 17β-estradiol (Cat. no. E4389), 100 µM cysteamine (Sigma–Aldrich, Cat. no. M6500), and 10% FBS (Gibco, Cat. no. GIB-16000–044) in a humidified atmosphere of 5% CO₂ at 38.5 °C.

Sperm preparation, in vitro fertilization (IVF) and in vitro culture of embryos (IVC)

Motile spermatozoa were selected by the Percoll gradient method. Briefly, frozen-thawed cattle semen at 35 °C was filtered by centrifugation on a Percoll discontinuous gradient (45–90%) at 1680 rpm for 15 min. To produce the 45% Percoll solution, 1 mL of capacitation-TALP medium was added to 1 mL of 90% Percoll. The sperm pellet was washed two times by the addition of 3 mL of capacitation-TALP medium and subsequent centrifugation at 1680 rpm for 5 min. Washed, motile spermatozoa were used for IVF. Spermatozoa ($1-2 \times 10^6$ sperm/mL) were incubated with mature oocytes for 18 h in 50 μ l microdrops of IVF-TALP medium covered with mineral oil (Nidacon, Cat. no. NO-100) in humidified atmosphere of 5% CO₂ at 38.5 °C. After 18 h of co-incubation, cumulus cells were removed from presumptive zygotes. The zygotes were cultured in a two-step chemically defined culture media [reference] that was covered in mineral oil in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂ at 38.5 °C.

Microinjection

When performing microinjection, Cas9 mRNA (Sigma–Aldrich, Cat. no. CAS9MRNA) and sgRNA were divided into 4 groups to find the most appropriate concentration (CB; only TE microinjection, RNA1X; Cas9 mRNA: 100 ng/ μ l, sgRNA: 50 ng/ μ l, RNA2X; Cas9 mRNA: 200 ng/ μ l, sgRNA: 100 ng/ μ l, RNA 4X; Cas9 mRNA: 400 ng/ μ l, sgRNA: 200 ng/ μ l). After 18 h of IVF, presumptive zygotes were injected with Cas9 mRNA and sgRNA synthesized by GeneArt Precision gRNA Synthesis Kit (ThermoFisher, Cat. no. A29377) for *MSTN* using a microinjector machine (Eppendorf, Femtojet®). The amount of injected Cas9 mRNA and sgRNA for *MSTN* were different each group (Fig 1 A-b). After 7 days of microinjection, pre-implantation stage embryos were collected and the mutation of *MSTN* was observed in vitro or transferred in vivo into a surrogate cow.

Primary cell culture

Primary cells were obtained by biopsy of the ear skin of calves. The ear skin was chopped into small pieces with a sterile scalpel and then washed several times and incubated at 38.5 °C for 4–18 h in Hank's Balanced Salt Solution (Gibco, Cat. no. 14175095) supplemented with collagenase (Collagenase type I, Gibco, Cat. no. 17–100–017). The following day the dispersed cells were washed several times in DMEM (Gibco, Cat. no. 21068028) and cultured in DMEM supplemented with 10% fetal calf serum (Gibco, Cat. no. GIB-11150–059), 1% penicillin/streptomycin (Gibco, Cat. no. 15140148), 1% non-essential amino acids (Gibco, Cat. no. 11140050), and 100 mM β -mercaptoethanol (Sigma–Aldrich, Cat. no. M3418).

Detection of *MSTN* gene mutation

Genomic DNA from transgenic primary cells was extracted using a DNA extraction kit (Qiagen, Cat. no. 69504). The *MSTN* primer was designed using PRIMER3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>, Supplementary Table 1), and the target sequence was amplified by PCR (94 °C for 5 min, 35–40 cycles of 94 °C for 20 sec/57 °C for 30 sec/72 °C for 35 sec, and 72 °C for 5 min). The PCR product from each sample was assessed using the T7E1 assay (Toolgene, Cat. no. TGEN_T7E1) to detect *MSTN* mutations.

Gene expression by real-time PCR

Total RNA was extracted from primary cultured cells using a RNeasy® Mini Kit (Qiagen, Cat. no. 74106), and complementary DNA was synthesized from 1 μ g of RNA using the RNA to cDNA EcoDry™ Premix (Takara, Cat. no. 639543). Gene expression assay was conducted using SYBR Green on QuantStudio 3 (Applied Biosystems, Model no. A28132), and relative cycle threshold (CT) values were normalized by GAPDH. The primers used in this study are listed in Supplementary Table 2.

Embryo transfer and pregnancy diagnosis

Blastocysts were stored in PBS supplemented with 20% FBS. A single blastocyst was transferred on day 7 (estrus = day 0 = day of fusion) to the uterine horn of each recipient animal using a non-surgical transcervical method. Pregnancy detection was performed on day 50 post estrus using rectal palpation and ultrasonography. Pregnant cattle were checked by rectal palpation and ultrasonography at regular intervals thereafter.

Targeted deep sequencing

Target sites were first amplified to a size of ~500 bp from extracted genomic DNA using KAPA HiFi HotStart DNA polymerase (Roche, Cat. no. #KK2502) according to the manufacturer's protocols. Then, amplicons were amplified again to a size of ~230 bp, after which the amplicons were amplified using TruSeq HT dual index-containing primers to add adaptor and index sequences for Illumina sequencing platforms to each sample [20]. Primers used in this study are listed in Supplementary Table 3. Pooled PCR amplicons were purified using a PCR purification kit (MGmed) and sequenced on a MiniSeq (Illumina) with paired-end sequencing systems (2x150 bp). Cas-Analyzer (<http://www.rgenome.net/cas-analyzer/#!>) was used to quantify the indel frequencies from deep sequencing data [20].

Analysis of *MSTN* off-target effect

The potential off-target effects caused by CRISPR–Cas9 in the three *MSTN* mutant calves were assessed using Cas-OFFinder software (<http://www.rgenome.net/cas-offinder/>). This software offers a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. In the *MSTN* target site, candidates of five loci targeting the whole gene of cows were found by setting the mismatch number to 3. A primer targeting each sequence (Supplementary Table 1) was prepared to confirm the off-target effect through T7E1 assay.

Blood analysis

To evaluate the general health status in calves, 5 mL of whole blood was collected from the jugular vein for complete blood count (CBC) analysis (Hemavet 950, Drew Scientific, USA) and serum chemistry analysis (BS-400, Mindray, China)s. The animals were also monitored by a veterinarian to assess their general health condition.

Statistical analysis

All data are presented as mean \pm SEM. Newman–Keuls Multiple Comparison Test and Tukey's test in a one-way ANOVA were used to evaluate statistically evaluate differences in embryo development, frequency of mutagenesis, and mRNA expression level. A value of $P < 0.05$ was considered statistically significant. All analyses were performed using the GraphPad Prism program (version 5.01).

Results

Validating single guide RNA for *MSTN* in bovine fibroblast cells

In bovine fibroblast cells, the selected sgRNA caused a mutation on the target site. The mutation on *MSTN* was detected by T7E1 assay (data not shown) and sanger sequencing. Though the sanger sequencing results, various types of mutation pattern were observed (Fig 1B). These results show that the selected sgRNA worked well on the *MSTN* target site.

Developmental competence and mutation efficiency on microinjection mediated gene editing

To determine optimal conditions, including Cas9 mRNA, and sgRNA concentrations for producing mutations in bovine blastocysts, four experimental conditions were used, then developmental competence and mutation efficiency were investigated in the blastocyst stage following each condition (Fig 1C, D, and E). There was no significant difference in cleavage rate across all 4 groups (data not shown). However, in the RNA4X group a diminished blastocyst formation rate was observed in comparison to the control group (Fig 1D). In terms of mutation rate, the RNA2X group ($81.3 \pm 17.2\%$) had the highest mutation compared the other 3 groups (WT: 0%, RNA1X: $33.3 \pm 16.0\%$, RNA4X: $50.0 \pm 28.5\%$) (Fig 1E). Thus, the RNA2X condition was selected as the optimal condition for the further production of *MSTN* mutated cattle.

Production of *MSTN* mutated Korean beef cow

The aim of this study was to produce *MSTN* mutated Korean beef cattle. Microinjection was performed on fertilized embryos under RNA2X conditions and cultured blastocysts were used for embryo transfer. The

experiment was repeated four times and 595 oocytes were used to generate mutant embryos. The cleavage rate was $59.0 \pm 21.0\%$ and 86 blastocysts ($14.5 \pm 14.0\%$) were produced. A total of 26 blastocysts with high quality morphology were transferred to surrogate mothers (one blastocyst per recipient). Some of leftover blastocyst ($n = 28$) were used to assess the mutation occurrence on *MSNT* and it was determined to be $71.6 \pm 44.3\%$.

After embryo transfer, 19 of the 26 surrogates were pregnant and 2 of the 19 fetuses were absorbed in the middle of pregnancy. A total of 17 calves (I.D.: #1 ~ #17) were live-born and one calf has a stillbirth (#14) from dystocia. Deep sequencing analysis showed mutations in 3 out of the 17 animals (Fig. 2A). Their *MSTN* mutation rates were 10.5, 45.4, and 99.9% for #6, #14, and #17, respectively. In addition, *MSTN* mutation was detected in #17 by T7E1 assay and there were no off-target effects (Fig. 2C). Real-time PCR was conducted to assess the *MSTN* RNA level of primary cells from #17 and #14. In both individuals the level of *MSTN* RNA was significantly decreased with wild type (Fig. 2D). Especially in #17 with the -12 deletion, enhanced muscle growth was observed compared with wild type calf (Fig. 2E). There were no abnormal values in a blood test performed to evaluate the general health status of 5 offspring (#4, #6, #7, #9 and #17) including the knockout one (#17) at 8-months-old (Table 1).

Discussion

CRISPR-Cas9 mediated genome editing is a powerful biological technology that has widespread application. Its application to livestock has been slow [21]. In this study, by applying genome editing without the integration of the transgene, an effective knockout condition was established at the embryo level and live-born edited offspring were produced. The *MSTN* knockout was used for proof of concept because of its clear double-muscling phenotype.

The *MSTN* gene consists of three domains (a signal sequence, a pro-peptide, and a mature-region). After transcription, translation, and two cleavage events (pro-peptide convertase by furin and tolloid protease by BMP-1 metalloprotease), the released mature MSTN protein dimer regulates the inhibition of skeletal muscle growth [22]. In more detail, the first cleavage occurs at the 266th position by Furin, followed by cleavage at 76th by BMP-1/Tolloid metalloproteinase, and finally, the released active MSTN protein dimer binds to the receptor (ActRIIB), resulted in inhibition of muscle growth [22-24]. Two representative cattle breeds, Belgian Blue, and Piedmontese, show natural mutations in this gene, 11 bps deletion, and one base mutation on mature *MSTN* domain, respectively. These cattle breeds phenotypically indicate that mature *MSTN* domain mutations contribute to muscle growth [5]. To mimic or reproduce those natural mutations using genome editing technologies, in a previous study, ZFNs disrupted exon1 locus (Signal Sequences region), subsequently, the mature *MSTN* domain was broken [25]. In another study, the mature domain locus via TALEN was directly targeted and mutated in microinjected embryos [19]. In both studies, the phenotype was observed after mutation of the mature locus of *MSTN*.

In our study it was assumed that disruption of the mature *MSTN* locus might occur by applying effective sgRNA on the pro-peptide locus using CRISPR-Cas9. Microinjected embryos were transplanted, and the muscle outgrowing phenotype was observed in one calf (#17). Thus, we thought that gene editing on the pro-peptide locus region worked well and predicted that the sequence of mature domain locus might be mutated by CRISPR-Cas9. However, one interesting finding was observed as a result of sequencing. This was an in-frameshift (-12bps deletion) knockout in the target locus that did not disrupt the mature *MSTN* domain region or amino acids of two cleavage regions. In other words, the 266th-, and 76th- amino acids for furin and proteinase were respectively conserved. Thus, the active MSTN protein dimer may be formed and muscle production is suppressed normally, and finally a wild type offspring should be born. Interestingly, the typical phenotyping (muscle outgrowth) was observed in #17 calf and the expression of *MSTN* mRNA was decreased (Figure 2). Because there have been no reports of phenotyping because of this type of mutation, it is hard to explain why this phenomenon occurred. One possibility is that the 156-160 position can be thought of as another molecular biological function in addition to the previously known two cleavage events. Similarly, in-frameshift mutation of the *MSTN* pro-peptide in mice showed a muscle gain phenotype [26]. Importantly, the blood test results of the mutated calves were normal (Table 1) and the calves showed no issue in their

general health. In the future, we will monitor the growth, including germline transmission, and investigate how this mutation may have affected the function of *MSTN*.

Microinjection commonly results in mosaic F0 founder animals that are then screened for the exact knock-out/knockin in the F1 generation following subsequent breeding. This technique is very effective in rodent experiments but is not suited to cattle because of their long gestational periods and single pregnancies. A cattle F0 and F1 system would take more than 3 years and require high costs. Consequently, most genome edited cattle are produced using a SCNT approach. However, live, healthy calf offspring are limited when SCNT is employed because of abnormal reprogramming during embryogenesis. In our study, microinjection was used to produce live, healthy genome edited calves. Randomly selected blastocysts were analyzed in vitro by sgRNA/Cas9 mRNA and a $81.3 \pm 17.2\%$ knockout efficiency rate was found. Embryo transfer was performed and a lower *MSTN* mutant cow generation rate of 17.6% was found in vivo. It is possible that non-mutated blastocysts were selected during the randomly selected process. In the future, to improve the efficiency of producing mutated offspring a portion of the blastocysts could be biopsied prior to transfer to identify possible mutations [27].

Genotyping analysis showed another interesting result. When mRNA of sgRNA and Cas9 was introduced into cells and embryos (blastocysts) various mutant pattern (-12, -10, -3, -2, -1, +1; Figure 1B) were shown, but only one mutant pattern (-12bps) was observed in genome edited calves. It is difficult to explain why only one pattern is observed in all *MSTN* mutated calves. One possible theory is that the cells with the other mutated pattern may be embryonic lethal at some time after the point of embryo transfer. Future studies will focus on improving in vitro and in vivo efficiency.

In conclusion, we demonstrated, for the first time, that microinjection of Cas9 mRNA and sgRNA for *MSTN* into in vitro fertilized embryos can produce live, genome edited, Korean beef calves—including one calf with biallelic mutation. These calves will be served as a model for the future development of CRISPR–Cas9 technology in the agricultural industries.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Table 1. Blood analysis in the offspring

Blood items	References	Offspring	Offspring	Offspring	Offspring	Offspring
		#4	#6	#7	#9	#17
WBC	4.0 ~ 12.0($10^3/\text{mm}^3$)	7.02	8.80	8.16	10.36	8.20
Neutrophil	0.6 ~ 4.1($10^3/\text{mm}^3$)	3.12	4.36	3.55	5.79	3.66
Lymphocyte	2.5 ~ 7.5($10^3/\text{mm}^3$)	2.42	2.53	2.65	3.48	3.30
RBC	5.0 ~ 10.0($10^3/\text{mm}^3$)	8.14	10.09	9.56	8.83	8.62
HGB	8.0 ~ 15.0 (g/dL)	11.20	12.80	11.60	11.20	10.70
HCT	24.0 ~ 46.0 (%)	26.30	29.30	27.70	25.10	27.20
PLT	200 ~ 800($10^3/\text{mm}^3$)	441.00	286.00	431.00	352.00	395.00
AST	53 ~ 162	55.00	78.00	63.00	75.00	68.00
ALP	77 ~ 265	209.00	321.00	331.00	390.00	343.00
BUN	10 ~ 25	21.00	12.00	13.00	12.00	12.00
Creatinine	0.4 ~ 2.0	0.90	1.10	0.90	1.00	1.70
Total Protein	7.2 ~ 9.0	7.40	8.30	8.20	7.90	7.60
Albumin	3.2 ~ 4.2	3.80	4.20	4.10	4.00	4.10
Ca (mg/dl)	8.3 ~ 10.4	9.40	9.55	9.77	9.35	9.99
P (mg/dl)	4.2 ~ 7.7	9.02	7.76	9.64	9.22	9.23
Total Cholesterol	73 ~ 280	144.00	112.00	110.00	104.00	152.00
Glucose	31 ~ 77	78.00	84.00	90.00	75.00	87.00
Triglyceride	7 ~ 323	15.00	10.00	14.00	12.00	30.00
Total Bilirubin	0.01 ~ 0.5	0.029	0.026	0.031	0.023	0.028

Figure legends

Figure 1. Efficient production of *MSTN* mutant blastocysts. A) Schematic design for *MSTN* target site (a) and graphical illustration on analyzing microinjected embryos (b) The red letters and line represent *MSTN* target site, and the green letters represent PAM sequence. B) *MSTN* mutation sequence pattern on pooled cells after transfection with sgRNA and Cas9 protein. C) Representative pictures of bovine blastocyst on day 7 for each experiment condition after microinjection (WT: microinjection with Tris-EDTA buffer, RNA1X; Cas9 mRNA: 100 ng/ μl , sgRNA: 50 ng/ μl , RNA2X; Cas9 mRNA: 200 ng/ μl , sgRNA: 100 ng/ μl , RNA 4X; Cas9 mRNA : 400 ng/ μl , sgRNA: 200 ng/ μl); D). The blastocyst formation rate after microinjection with each condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. E) *MSTN* mutation rate of day 7 blastocysts for each condition.

Figure 2. Birth of *MSTN* mutated Korean beef calves. A) Mutation rate of fetuses (labeled #1–17) on *MSTN* target site by deep sequencing. B) Mutation assay using T7E1 assay for one calf (#17) (WT: wild

type, NC: negative control, PC: positive control, W- :T7E1 assay without wild type genomic DNA, W+: T7E1 assay with wild type genomic DNA). C) Analysis of off-target effects on five candidate genes. D) Relative expression of MSTN mRNA. The bar graph represents the fold changes in mRNA levels, and the error bars show SEMs (n=3). *P < 0.05, **P < 0.01, ***P < 0.001. E) Representative pictures of a biallelic knockout calf (#17) along with age (a: 1 month, b: 2 months, c: 3 months, and d: 4 months).

