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Generation and characterization of *MSTN*-disrupted fibroblast clones in buffalo, goats, and sheep using CRISPR

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Myostatin (MSTN) is a negative regulator of muscle growth, whose disruption results in enhanced muscle mass in farm animals. This study utilized CRISPR-based genome editing technology to generate *MSTN* gene-edited fibroblast cell clones from buffalo, goat, and sheep. The exon 1 of *MSTN* gene in buffalo, goat, and sheep fibroblast cells was targeted using CRISPR/Cas9. Single-cell clones were isolated using a microscope-guided single-cell picking method and subsequently screened. We successfully established 9, 10, and 12 single-cell-derived colonies from buffalo, goat, and sheep fibroblasts, respectively. We have noted that sheep fibroblasts exhibited the highest cell attachment and proliferation rates (83.67% and 78.04%, respectively), followed by goat and buffalo fibroblasts. Buffalo fibroblasts showed the lowest rates, likely due to species-specific cellular characteristics. Gene-editing efficiency varied among species, with buffalo at 33.33%, goat at 30%, and sheep at 16.67%. The clones exhibited diverse edit types, including homozygous bi-allelic and mono-allelic deletions. Alpha Fold prediction indicated that all alleles contained non-multiple-of-three deletions, which resulted in frame shift mutations and truncated MSTN proteins. This study successfully produced MSTN gene-edited fibroblast cell clones from buffalo, goats, and sheep. These clones hold significant potential for application in somatic cell nuclear transfer to produce *MSTN*-edited embryos and farm animals.

Keywords: Buffalo, Goats, Sheep, Single-Cell Clones

Introduction

The increasing demand for meat and other animal products has prompted significant advancements in livestock production technologies. Among these, genome editing has emerged as a transformative tool, allowing precise modifications to enhance both production and health traits[1]. The CRISPR tools, in particular, have gained prominence for its efficiency, versatility and affordability[2]. Genome editing techniques have been employed to edit genes associated with various desirable traits, such as the myostatin (*MSTN*) gene for improved meat production[3-7], the *PRLR* gene for enhanced milk production[8], *BLG* gene for allergen free milk production[9-11], and the *FGF5* gene for superior wool and fiber quality[12-14]. In addition to the production traits, genome editing has also been utilized to enhance disease resistance by targeting key genes like *CD163*[15], *CD46*[16], *CD18*[17], and *NRAMP1*[18,19]. In addition, genome editing has been applied to improve the climate resilience in livestock species and to ensure the welfare of animals and handlers, such as through the creation of hornless cattle[20,21].

To generate gene-edited livestock, two main reproductive bio-techniques are employed, namely somatic cell nuclear transfer (SCNT) and zygote microinjection. SCNT is particularly favored due to its numerous advantages, including the ability to pre-screen edited cells, pre-determine the sex of the animal, avoid mosaicism, and facilitate extensive optimization experiments owing to the availability of somatic cells in bulk quantities[22]. However, a critical and challenging aspect of SCNT is the establishment of single-cell clones having targeted modifications. In the context of meat production, the *MSTN* gene, which regulates muscle growth, has been a primary target for genome editing[23]. Disabling this gene has significantly improved muscle growth and meat quality[4-6]. Recently, our laboratory has successfully used the CRISPR tool to produce cloned buffalo embryos having the editing of *MSTN* gene[7]. In 2023-24, India exported the farm animal products to the world for the worth of4,543.52 USD millions, in which the buffalo meat is the major contributor (3740.53 USD millions), and sheep and goat meat contributed 77.68 USD million (https://apeda.gov.in/apedawebsite/six_

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head_product/animal.htm, accessed on 24th July, 2024). Due to the enormous utility of buffalo, goat and sheep for meat production in India, this study aims to establish single-cell clones having *MSTN* gene edits in these species. In future, the established cells can be efficiently used in SCNT to produce gene-edited animals with improved meat production traits, aiming to support the meat production industry in India.

Materials and Methods

Establishment of fibroblast cells

Tail tissue biopsies from female buffalo, goat, and sheep were collected to establish the primary fibroblast cell cultures. This study adheres to the standards of ethical conduct as per the institutional guidelines. The tissue samples were transported to the laboratory in Dulbecco's Phosphate Buffered Saline (DPBS) maintained at 4°C. Upon arrival, the samples were sterilized by washing twice in 70% ethanol, followed by a rinse in DPBS. The tissue pieces were then trimmed to approximately 1 mm^3 size and placed in a holding medium composed of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin. Four to five pieces were transferred to each 25 cm² cell culture flask and incubated in a CO_2 incubator for 4-5 hours to allow the explants to adhere. After the initial incubation period, cell culture medium containing DMEM, 20% FBS, 1% non-essential amino acids (NEAA), and 50 µg/ml gentamicin was added to the flasks. The culture flasks were then placed in the incubator undisturbed for 4-5 days to facilitate cell outgrowth from the explant tissues. Once significant cell outgrowth was observed, the cells were passaged using 0.25% trypsin and subsequently cryopreserved using a slow freezing method.

Design of sgRNA

To establish knockout single-cell clones for the muscle growth-controlling gene *MSTN* in buffalo, goat, and sheep, the sgRNA was designed using the CHOPCHOP online tool (https://chopchop.cbu.uib.no/). The design prioritized targeting the first exon of the *MSTN* gene, ensuring the highest on-target score. The selected sgRNA sequence for *MSTN* targeting was 5'-CAATCAGTTCCAGGAGTGG-3', which was commercially synthesized. The produced sgRNA oligonucleotides were then utilized to create the gRNADNA template using the GeneArt™ Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Cat #A29377), which was subsequently used for in vitro transcription to synthesize the sgRNA. The resulting sgRNA was then purified and quantified for use in genome editing experiments.

Transfection of cells

To introduce the CRISPR/Cas9 ribonucleoprotein (RNP) complex into fibroblast cells, electroporation was employed. For electroporation, fibroblast cells were cultured to 60-70% confluence in a 25 cm^2 flask and then detached using the trypsin-EDTA solution. The detached cells were resuspended in 100 µL of transfection buffer. A separate mixture (10 µL), containing 2 µg of sgRNA and 10 µg of Cas9 protein, was prepared. This mixture was added to the cell suspension, and the components were mixed thoroughly and incubated for 5 minutes at room temperature. Following the incubation, the cell suspension was transferred to an electroporation cuvette with a 4 mm gap. Electroporation was performed using the BTX 2001 Lite electroporation machine, applying a single square pulse of 300 V for 10 ms. Post-electroporation, the cells were immediately seeded into a T-25 cm² cell culture flask containing 3-4 ml of cell culture media and incubated for 72 hours to allow recovery and expression of the CRISPR/Cas9-mediated edits.

Establishment of single cell clones

72 hours post-electroporation, the fibroblast cells were detached from the culture flask. A small volume (5-8 µL) of the cell suspension was transferred into a 35 mm cell culture dish containing 3 mL of culture media. Using a glass pipette, individual cells were manually picked up and each single cell was transferred to an individual well of a 96-well plate containing cell culture media supplemented with epidermal growth factor (EGF, 10 ng/mL) and fibroblast growth factor (FGF, 1 ng/mL). The cells were then incubated and allowed to grow until they reached 70-80% confluence. Upon reaching confluence, the colonies from each well of the 96-well plate were transferred to a 48-well plate. After the cells in the 48-well plate reached confluence, the cell colonies were transferred to separate wells in a 6-well plate. The cells were allowed to proliferate until a sufficient number of cells were obtained for each clonal population.

Screening of single cell clones

To determine the type of editing event (mono-allelic or bi-allelic) in the established single cell clones, TA cloning of the target region was performed. The *MSTN* gene target region from each single-cell clone was PCR amplified, and then cloned into the pJET1.2 vector using the CloneJET PCR cloning kit (Thermo Fisher

Scientific, Cat # K1232). The ligated products were transformed into *E. coli* competent cells. For the transformation process, the competent cells were first incubated on ice for 20 minutes. Subsequently, 2 µL of the ligated plasmid was added to the competent cells, and the mixture was incubated on ice for an additional 25 minutes. A heat shock was then applied at 42ºC for 90 seconds, followed by incubation on ice for 10 minutes. After the heat shock, 250 µL of Luria Broth (LB) medium was added to the transformation mixture, which was then incubated on a shaker at 200 rpm and 37ºC for 45 minutes. The bacterial culture was spread on LB agar plates containing ampicillin to select for transformed colonies. Plasmids were isolated from 6 to 10 *E. coli* colonies using a plasmid extraction kit (Thermo Fisher Scientific, Cat # K210011). The isolated plasmids were sequenced using Sanger sequencing. The sequencing data were then analyzed to determine the type of editing event (mono-allelic or bi-allelic) in each single-cell clone.

Fate analysis of edited sequences

The nucleotide sequence (OK913670.1, OK913674.1, and OK913678.1) and their corresponding amino acid sequence (UWL52252.1, UWL52256, and UWL52260) of the wild-type *MSTN* gene were sourced from the NCBI database. In this study, exon 1 of the *MSTN* gene was edited and their probable coding sequences were generated using the ExPASy translation tool (https://web.expasy.org/translate/). The predicted open reading frames (ORFs) of the edited and the wild type nucleotide/amino acid sequences were compared through multiple sequence alignment using T-Coffee (https://www.ebi.ac.uk/Tools/msa/tcoffee/). The 3-D protein structures of the edited and the wild type MSTN proteins were predicted using the AlphaFold2, a highly efficient deep learning and artificial intelligence (AI)-based tool (https://alphafold.ebi.ac.uk/), and the resulting predicted structures were visualized using the PyMOL molecular graphics system, Version 2.0, by Schrödinger LLC (https://pymol.org/2/).

Results and Discussion

CRISPR/Cas is a widely recognized genome editing tool in genetic engineering due to its site-specificity, effectiveness, multiplexing capability, and easy customization[24]. This technology has been employed to introduce desirable traits in farm animals such as increased muscle mass[6], beta-lactoglobulin-free milk[9], enhanced fiber production[14], and disease resistance[25]. MSTN also known as GDF8, is a negative regulator of muscle growth. CRISPR-based editing of *MSTN* has led to increased muscle mass production in farm animals[23]. In this study, we aimed to generate CRISPR-mediated *MSTN* gene-edited single-cell clones of buffalo, goat, and sheep (Fig. 1).

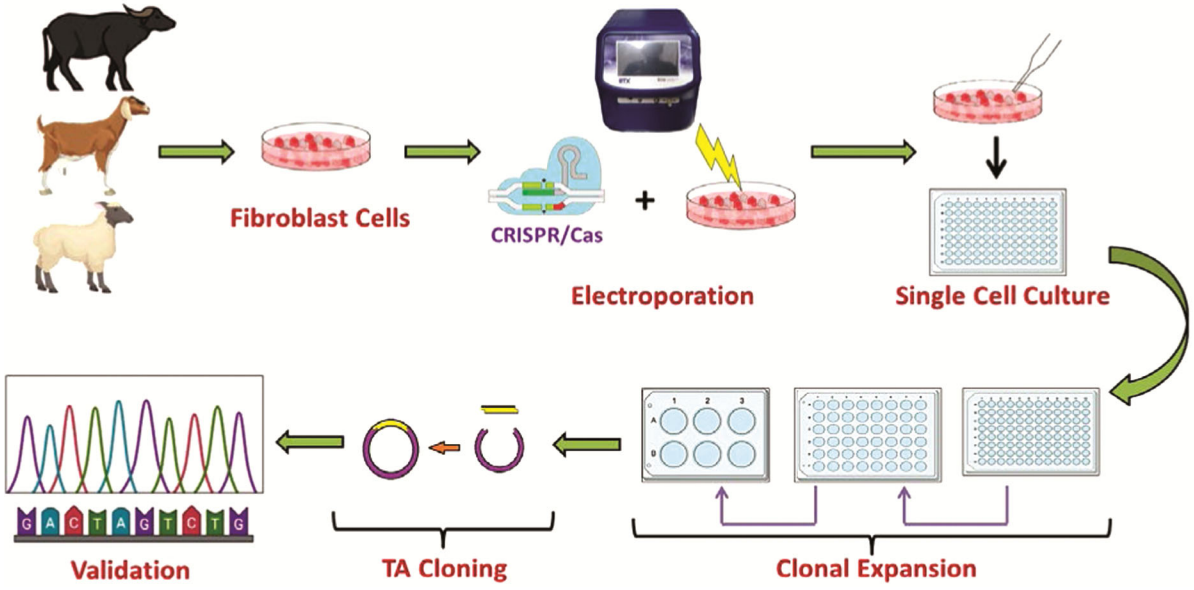

Fig. 1 — Schematic presentation of the experimental activities. Fibroblast cells from buffalo, goats, and sheep were isolated and electroporated with a ribonucleoprotein complex (RNP) consisting of sgRNA and Cas9, targeting the first exon of the *MSTN* gene. After electroporation, the cells were individually plated in a 96-well plate (one cell per well) to allow for clonal expansion. The clones were then screened for *MSTN* gene edits using TA cloning followed by Sanger sequencing. The edited sequences were subsequently analyzed to predict the protein structure using the AlphaFold software. MSTN, myostatin.

To generate the cell clones, we utilized the RNP approach, which has demonstrated higher target specificity compared to other methods[26]. For the delivery of CRISPR components, we employed electroporation, an effective transfection strategy for buffalo fibroblast cells, which has been successfully used to produce gene-edited cell clones[7,27,28]. As a result, we successfully generated MSTN gene-edited cell clones of fibroblast cells from all the three species.

Achieving single-cell clones from electroporated mixed-cell populations is a critical aspect of genome editing experiments. This ensures the generation of genetically identical cells, which is essential for precise and reliable experimental outcomes. The initial step in single-cell cloning involves isolating individual cells from a larger mixed-cell pool. The single cell culture is crucial for establishing pure clones, and one of the most popular methods for this purpose is limiting dilution[29]. Limiting dilution is favored due to its cost-effectiveness and convenience; however, it has notable drawbacks. This method often requires a significant amount of culture media and carries the inherent risk of inadvertently placing multiple cells into a single well, which can compromise the integrity of the single-cell clones[30]. In our laboratory, we utilized a microscope-guided method for cell picking and successfully established the single cell culture for all the three species (Fig. 2). This method has proven to be highly efficient in terms of both speed and accuracy, significantly reducing the likelihood of generating clones derived from multiple cells. Recently, Tara *et al.*[28] demonstrated the effectiveness of this approach by successfully establishing fifteen single-cell clones of buffalo fibroblasts. Utilizing this methodology, we successfully established 9, 10, and 12 single-cell-derived colonies from buffalo, goat, and sheep fibroblasts, respectively (Table 1). This accomplishment indicates the effectiveness of the microscope-guided method across different species.

To further evaluate the efficiency of this single-cell culture approach, we recorded the cell attachment and proliferation rates for each species. The attachment rates for buffalo, goat, and sheep fibroblasts were 66.70%, 77.08%, and 83.67%, respectively. The results indicated that sheep fibroblasts exhibited the highest attachment rate, followed by goat and buffalo fibroblasts. The relatively lower attachment rate observed in buffalo cells may be attributed to the

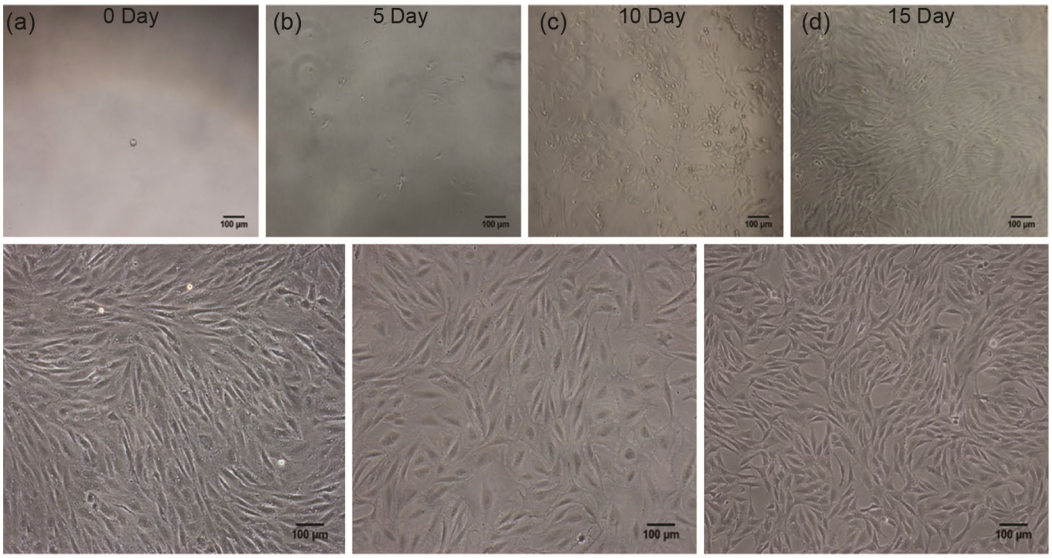

Fig. 2 —Establishment of single cell clonal population. (A-D) Sequential images showing the culture of a single cell at day 0, day 5, day 10, and day 15. Confluent *MSTN*-edited single cell derived clonal population of buffalo (E), sheep (F), and goat (G). Scale Bar: 100 µm

Table 1 — In vitro growth status of single cells of buffalo, goat, and sheep

Species	Single cells seeded in 96-well plate	Cell attachment n (%)	Cell proliferation n (%)	Screened cell clones n	Edited cell clones n (%)
Buffalo	48	32 (66.70)	21 (65.63)	9	3 (33.33)
Goat	48	37 (77.08)	28 (75.67)	10	3 (30)
Sheep	49	41 (83.67)	32 (78.04)	12	2 (16.67)

inherent nature of the cells from this species, which might present unique challenges in adhering to culture surfaces. In addition to cell attachment, we also assessed the proliferation rates of the single-cell cultures. The proliferation rates for buffalo, goat, and sheep fibroblasts were 65.63%, 75.67%, and 78.04%, respectively. Similar to the attachment rates, buffalo cells demonstrated the lowest proliferation rate, while sheep cells showed the highest. The reduced proliferation rate in buffalo fibroblasts could be linked to species-specific cellular characteristics that affect their growth dynamics in culture conditions. The microscope-guided method for cell picking has proven to be a robust technique for establishing single-cell-derived colonies in farm animals. Despite the lower attachment and proliferation rates observed in buffalo fibroblasts, the method remains effective, showcasing its potential applicability to various cell types and species.

The validation of gene-editing events is crucial as it helps understand the nature of INDELs induced during cellular repair pathways. In this study, we determined the nature of edits using TA cloning and Sanger sequencing. For buffalo, nine single-cell clones were screened, out of which three clones (B1, B2, and B3) were found successfully edited, resulting in a 33.33% editing efficiency. The specific alterations in these clones varied: colonyB3 exhibited a homozygous bi-allelic (−/−) edit with a 16 base pair (bp) deletion in each allele. In contrast, Clones (clones) B1 and B2 showed mono-allelic (−/+) edits with 1 bp and 2 bp deletions, respectively (Fig. 3a). In goats, the analysis of ten single-cell clones identified three edited clones, resulting in a 30% editing efficiency. The types of edits observed included homozygous bi-allelic (−/−) edits in Clones (clones) G2 and G3, each characterized by a 2 bp deletion. Clone G1 displayed a mono-allelic (−/+) edit with a 2 bp deletion (Fig. 3b). For sheep, the screening revealed two edited clones out of twelve, resulting in a 16.67% editing efficiency. One clone (S2) exhibited a heterozygous bi-allelic (−/−) edit with a 2 bp deletion from each allele, alongside one allele showing a single base pair replacement. Another clone (S1) demonstrated a mono-allelic (−/+) edit with a 2 bp deletion and one G to T base pair replacement (Fig. 3c). The editing efficiencies observed in this study are comparable to those reported in the literature. For instance, Bajwa et al [27] reported a 30% editing efficiency for the *ITGB2*

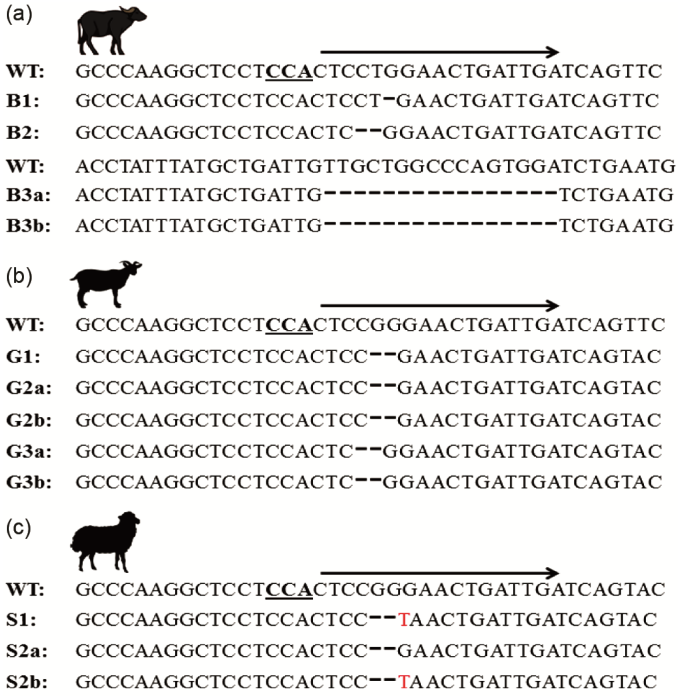

Fig. 3 — Sanger sequencing output of *MSTN* target site. In buffalo fibroblast clones (A), clone B1 is mono-allelic edited with a 1 bp deletion, clone B2 is also mono-allelic edited with a 2 bp deletion, and clone B3 shows bi-allelic editing with a 16 bp deletion in both alleles. In goat cell clones (B), clone G1 is mono-allelic edited with a 2 bp deletion, while clones G2 and G3 exhibit bi-allelic editing with a 2 bp deletion in both alleles. For sheep cell clones (C), clone S1 is mono-allelic edited with a 2 bp deletion and a 1 bp replacement, whereas clone S2 is bi-allelic edited with a 2 bp deletion and a 1 bp replacement in one of the alleles.

gene, and Dua *et al.*[7] reported a 25% editing efficiency for the *MSTN* gene in buffalo fibroblast cells, consistent with the buffalo results here. The results reflect the varied outcomes of CRISPR/Cas-induced double-strand breaks (DSBs) and subsequent repair mechanisms.

Gene editing technology allows precise modifications in specific gene(s) to investigate the functional consequences of these edits. This study delved into the consequences of *MSTN*-edited sequences on protein structure and function using bioinformatics tools. The *MSTN* nucleotide sequences from edited clones were translated into amino acid sequences using the ExPASy translation tool. The translated sequences were then aligned with the wild-type *MSTN* protein sequence to identify alterations, such as insertions, deletions, and substitutions, which could impact protein function. In our experiment, all alleles contained non-multiple-of-three deletions. Such deletions are particularly impactful as they cause frame shift mutations, leading to a shift in the reading frame during translation (Fig. 4a, 4c, 4e). This shift typically resulted in the incorporation of incorrect amino acids downstream of the mutation site and often introduces a premature stop codon, leading to early truncation of the protein. To predict the three-dimensional (3D) structure of the MSTN protein, we utilized Alpha Fold, a state-of-the-art tool that provides accurate protein structure predictions[31]. Alpha Fold takes into account various sequence alterations, including insertions, deletions, and substitutions, which are crucial for understanding how gene edits affect protein folding and function[32].The wild-type MSTN protein consists of 375 amino acids. However, frame shift mutations introduced by non-multiple-of-three deletions altered this composition.

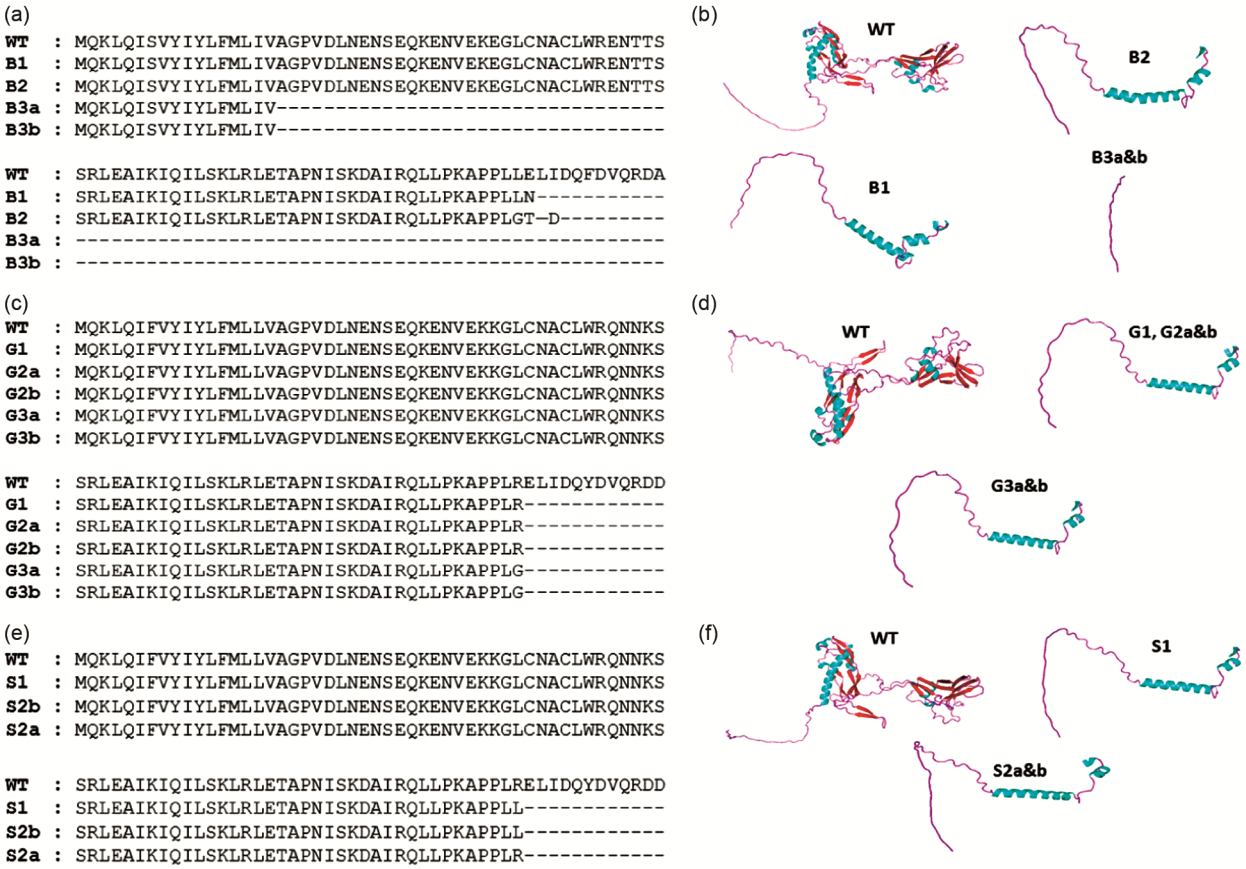

Fig. 4 — Protein Sequence and Structure Prediction Based on ExPasy and AlphaFold Tools. The buffalo cell clones, namely B1, B2, and B3a&b have early truncation after 89 amino acids, 90 amino acids, and 17 amino acids, respectively compared to the wild-type sequence which has 375 amino acids (A). The 3D-protein structures of the truncated proteins from clones B1, B2, and B3 are presented alongside their wild-type counterpart from buffalo (B). In case of goat single cell clones, G1, G2 and G3 exhibited truncation after 88 amino acids (C), and also predicted non-functional 3D-protein structures (D). The sheep clones predicted protein sequences reveal early truncation after 88 amino acids in clone S1 and after 88 amino acids in both alleles of clone S2 (E), and their predicted non-functional 3D-protein structures (F).

These mutations disrupted the normal reading frame, resulting in the synthesis of truncated proteins with significantly altered structure (Fig. 4b, 4d, 4f). In the case of *MSTN*, the frame shift and subsequent early truncation likely disrupt its normal regulatory functions. The wild-type *MSTN* protein acts as a negative regulator of muscle growth; thus, any structural alteration could impair its ability to bind to its receptors or other interacting proteins, leading to uncontrolled muscle development.

Conclusions

In conclusion, we successfully created CRISPR-mediated *MSTN* gene-edited fibroblast cell clones from buffalo, goat, and sheep. The microscope-guided single-cell picking method efficiently generated single-cell clones from electroporated cells. These clones have frame shift mutations that likely disrupt normal muscle growth regulation, making them ideal donors for SCNT applications to produce *MSTN*-edited embryos and animals. Our long-term goal is to combine genome editing with SCNT to produce *MSTN* gene-edited farm animals, which will enhance the meat production and advance the livestock improvement.

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Conflict of interest

The authors declare no conflict of interest.

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