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MOET-assisted CRISPR/Cas9-mediated MSTN genome editing in caprine embryos by electroporation and embryo transfer

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Abstract

The present study aimed to establish a CRISPR/Cas9-mediated approach for targeted modification of the myostatin (MSTN) gene in MOET-derived caprine presumptive zygotes using electroporation. An sgRNA targeting exon 1 of MSTN was complexed with recombinant Cas9 protein to form a ribonucleoprotein (RNP) complex. A total of 78 presumptive zygotes recovered laparoscopically from eight superovulated donor does were electroporated using three square-wave pulses at 25 V, 3 ms duration, and 100 ms intervals. Following *in vitro* culture, all embryos developed to the morula stage, with two reaching the blastocyst stage. T7E1 analysis of four randomly selected morulae revealed cleavage at the targeted locus in three embryos (75%). Four 4-cell embryos were transferred into a synchronized Sirohi recipient, resulting in pregnancy and recovery of two morphologically normal fetuses on Day 57. T7E1 analysis detected cleavage at the targeted locus in one fetus. These findings demonstrate the potential of integrating MOET with CRISPR/Cas9 RNP electroporation for targeted genome editing in caprine embryos.

Introduction

Goat production plays an important role in sustainable livestock farming, particularly in regions where goats contribute substantially to meat production, livelihood security, and utilization of marginal production environments. Improvement of economically important production traits in goats has traditionally relied on selective breeding and reproductive technologies. However, conventional breeding is constrained by the time required to achieve genetic improvement and by the availability of naturally occurring desirable alleles. Recent advances in genome editing have provided new opportunities to introduce precise genetic modifications associated with economically important traits while maintaining the reproductive potential of elite animals[1].

The myostatin (MSTN) gene, also known as growth and differentiation factor 8, is a member of the transforming growth factor-β superfamily and functions as a negative regulator of skeletal muscle growth[2]. Genetic disruption or reduced activity of MSTN has been associated with increased skeletal muscle development in several livestock species. Consequently, MSTN has emerged as an important candidate gene for genome editing based improvement of muscle related production traits. The generation of MSTN edited livestock therefore represents a promising application of genome editing for investigating gene function as well as developing animals with potentially enhanced muscle growth and development.

The CRISPR/Cas9 system has become one of the most widely used genome editing platforms because of its programmability, relative simplicity, and ability to introduce targeted DNA modifications[3]. In this system, a single-guide RNA (sgRNA) directs the Cas9 endonuclease to a complementary genomic sequence adjacent to an appropriate protospacer adjacent motif (PAM), where Cas9 generates a site-specific DNA double strand break. Repair of the induced DNA break, predominantly through endogenous DNA repair pathways, can result in insertion or deletion mutations and consequently disrupt the function

of the targeted gene. Delivery of CRISPR/Cas9 components into mammalian embryos is therefore a critical determinant of successful genome editing.

Several approaches have been developed for introducing genome editing components into embryos, including cytoplasmic or pronuclear microinjection and electroporation. Although microinjection provides direct delivery of editing reagents into individual embryos, it requires specialized equipment, considerable technical expertise, and manipulation of embryos one at a time. Electroporation provides an alternative approach in which transient permeabilization of the embryonic plasma membrane facilitates intracellular delivery of macromolecules[4]. The ability to process multiple embryos simultaneously makes electroporation particularly attractive for livestock embryo genome editing. Moreover, delivery of Cas9 protein pre-complexed with sgRNA as a ribonucleoprotein (RNP) complex provides a transient editing system in which the active editing machinery can be introduced directly into embryos without the requirement for expression of Cas9 from exogenous DNA.

The integration of genome editing with assisted reproductive technologies can further facilitate the production and evaluation of genetically modified livestock. Multiple ovulation and embryo transfer (MOET) enables the recovery of multiple *in vivo* derived embryos from genetically valuable donor animals and their transfer into synchronized recipients[5]. Compared with embryo production entirely under *in vitro* conditions, the use of MOET derived embryos provides an opportunity to combine established reproductive technologies with genome editing while retaining the advantages of *in vivo* embryo development before recovery. However, efficient genome editing of MOET derived caprine embryos, particularly immediately after recovery at the presumptive zygote stage, requires optimization of the delivery procedure while preserving embryo developmental competence.

The stage at which genome editing components are introduced is also important for determining subsequent editing outcomes. Editing at the presumptive zygote stage provides an opportunity for the introduced CRISPR/Cas9 machinery to act at an early developmental stage, potentially allowing the resulting genetic modification to be carried forward during subsequent embryonic development. Following genome editing, embryos may either be maintained *in vitro* for molecular evaluation or transferred into synchronized recipients to assess their developmental competence *in vivo*. Direct transfer of electroporated presumptive zygotes may provide a streamlined workflow by reducing additional manipulation and prolonged *in vitro* culture between genome editing and embryo transfer. Nevertheless, this study represents the first report of CRISPR/Cas9-mediated MSTN gene editing in MOET derived caprine presumptive zygotes using electroporation, followed by their transfer into recipient goats.

In this context, the present study aimed to establish a CRISPR/Cas9-mediated genome editing approach targeting the MSTN gene in MOET derived caprine presumptive zygotes using electroporation. The study further aimed to assess MSTN modification in *in vitro* developed embryos and to determine the feasibility of transferring electroporated presumptive zygotes into synchronized recipient does for subsequent *in vivo* development and molecular assessment of recovered fetuses.

Results

Recovery, electroporation and in vitro development of MOET derived presumptive zygotes

Across the eight MOET donor does, a total of 78 presumptive zygotes were recovered. All recovered presumptive zygotes exhibited normal morphology, characterized by an intact zona pellucida and homogeneous cytoplasm, and were considered suitable for subsequent CRISPR/Cas9-mediated genome editing. All presumptive zygotes were electroporated with the preassembled CRISPR/Cas9 RNP complex using the optimized parameters. Following electroporation, the embryos retained normal morphology without visible signs of degeneration, indicating preservation of embryo integrity (Fig. 2A, B).

Embryonic development was monitored morphologically, and all embryos maintained in *in vitro* culture progressed to the morula stage, with two subsequently developed to the blastocyst stage (Fig. 2C,D). Four morula-stage embryos were randomly selected for molecular analysis. Before gDNA extraction, the zona pellucida was removed by treatment with 0.25% pronase to facilitate cell lysis and improve the efficiency of gDNA isolation (Fig. 2E). Furthermore, randomly selected four 4-cell embryos (n = 4) were transferred into synchronized recipient does to evaluate their *in vivo* developmental potential (Fig. 2F).

gDNA was successfully isolated from individual morula stage embryos and subjected to WGA. Agarose gel electrophoresis confirmed successful WGA in all four morulae, yielding the expected amplification products for molecular analyses (Fig. 3A). Genome editing was subsequently evaluated using the T7E1 assay. Cleavage fragments indicative of CRISPR/Cas9-mediated mutations were detected in three of the four morulae (75%), confirming successful editing of the targeted MSTN locus following electroporation (Fig. 3B).

Embryo transfer, pregnancy establishment and fetal recovery

The 4 cell embryos (n = 4) were transferred laparoscopically into a synchronized Sirohi recipient doe. Pregnancy was confirmed by trans-rectal ultrasonography, which revealed viable conceptuses with distinct embryonic structures and detectable fetal heartbeats (Fig. 4A). The recipient doe subsequently died due to pneumonia at 57 days of gestation. Post-mortem examination enabled the recovery of two intact fetuses, Fetus 1 (male) and Fetus 2 (female), both of which appeared morphologically normal without any apparent developmental abnormalities (Fig. 4B–D). Fetus 1 (male) had a crown-rump length of 11.5 cm and a body weight of 28.67 g, whereas Fetus 2 (female) had a crown-rump length of 10.5 cm and a body weight of 27.49 g. The observed fetal morphology and morphometric characteristics indicated apparently normal development at the time of recovery and corresponding to the gestational age. These findings demonstrated successful pregnancy establishment and fetal development following the transfer of CRISPR/Cas9 electroporated caprine zygotes.

Molecular confirmation of genome editing in fetal tissues

gDNA was successfully isolated from ear tissue samples of both recovered fetuses and subjected to PCR amplification of the targeted MSTN locus. Agarose gel electrophoresis confirmed amplification of PCR products of the expected size in both Fetus 1 and Fetus 2 (Fig. 5A). The amplified products were subsequently subjected to the T7E1 assay to assess the presence of CRISPR/Cas9-induced sequence mismatches. Fetus 1 (male) exhibited cleavage fragments in the T7E1 assay, indicating the presence of mutations at the targeted MSTN locus. In contrast, no cleavage fragments were detected for Fetus 2 (female), indicating the absence of detectable genome editing at the targeted locus (Fig. 5B).

Discussion

The integration of genome editing with MOET offers a potential approach for introducing targeted genetic modifications into embryos recovered from genetically valuable donor animals. In the present study, CRISPR/Cas9 RNP mediated electroporation was incorporated directly into a MOET based caprine embryo workflow, with the electroporated presumptive zygotes either cultured *in vitro* or transferred immediately to a synchronized recipient. The results provide evidence that this approach can combine embryo manipulation with preservation of developmental competence and detectable modification of the target locus.

The developmental outcome of the cultured embryos provides further evidence supporting the suitability of the selected electroporation conditions. All electroporated presumptive zygotes progressed to the morula stage, and two embryos subsequently developed to the blastocyst stage. During the culture period, a set of four-cell embryos (n = 4) was selected and transferred to a synchronized recipient doe for assessment of *in vivo* developmental competence. The progression of electroporated embryos through successive stages of preimplantation development, including blastocyst formation, demonstrates the compatibility of the optimized RNP-electroporation approach with embryo developmental competence. Furthermore, the detection of T7E1 cleavage at the targeted MSTN locus in three embryos confirms successful CRISPR/Cas9-mediated modification of the target locus. Together, these findings highlight the potential of RNP-electroporation as an efficient and developmentally compatible strategy for targeted genome editing in MOET-derived caprine embryos, providing a promising platform for advancing precision breeding and functional genomic studies in goats.

The use of electroporation also offers practical advantages for integrating genome editing into MOET based embryo manipulation. CRISPR/Cas9 components have previously been introduced into caprine zygotes by microinjection[6], which enables direct delivery but requires individual manipulation of each embryo. Electroporation permits simultaneous treatment of multiple embryos and may therefore be more amenable to workflows in which several embryos are recovered from a donor. In the present study, RNP delivery was achieved in zona-intact presumptive zygotes using three 3-ms pulses at 25 V, without removal or weakening of the zona pellucida. Thus, the present findings support the feasibility of incorporating zona-intact electroporation into a MOET based genome-editing.

The use of a preassembled Cas9/sgRNA RNP further complements this strategy. Genome editing reagents can be delivered in different formats, including plasmid DNA, Cas9 mRNA with sgRNA, or preassembled RNP complexes[7,8]. Unlike DNA or RNA based approaches, RNP delivery provides Cas9 in its functional form, allowing genome cleavage without requiring intracellular transcription and translation of the nuclease[9]. Its transient intracellular activity may also provide a more restricted period of nuclease exposure compared with approaches that support prolonged expression. In the present study, detection of target-site modification in three of four randomly selected morulae demonstrates that the electroporated RNP remained functionally active during early embryo development.

Furthermore, an important feature of the present workflow was the sequential progression of electroporated presumptive zygotes through early *in vitro* development before embryo transfer. Following electroporation, the presumptive zygotes were cultured and allowed to progress to the 4-cell stage, after which four embryos were selected and transferred to a synchronized recipient doe for assessment of *in vivo* developmental competence. This approach enabled the embryos to undergo an initial phase of post-electroporation development prior to transfer, thereby providing an opportunity to select developmentally progressing embryos for subsequent *in vivo* evaluation. Following transfer, pregnancy was established, and two morphologically normal fetuses were recovered at 57 days of gestation, demonstrating the capacity of the electroporated embryos to sustain development following embryo transfer.

These findings indicate that the manipulation and electroporation procedure were compatible with development beyond the preimplantation stage. More importantly, T7E1 cleavage was detected at the targeted MSTN locus in one of the two recovered fetuses, providing evidence that detectable target site modification introduced at the presumptive zygote stage could be identified during subsequent fetal development. Thus, the present study extends the evaluation of the editing procedure beyond early embryo development to an *in vivo* developmental outcome.

The different molecular outcomes observed in the two recovered fetuses highlight the potential for embryo-to-embryo variation following CRISPR/Cas9-mediated genome editing. Although both transferred presumptive zygotes received the same RNP electroporation treatment, target-site modification was detected in Fetus 1 but not in Fetus 2. Variable editing outcomes have been reported across livestock embryos, including goats, sheep, pigs and cattle, and have been associated with differences in the timing of genome-editing activity during early embryonic development and the subsequent distribution of edited and unedited cell lineages[10,11].

To our knowledge, the specific combination of MOET-based embryo recovery, CRISPR/Cas9 RNP electroporation, transfer of manipulated presumptive zygotes, and subsequent detection of MSTN modification during fetal development has not previously been reported in goats. Overall, the present study demonstrates the feasibility of linking MOET based embryo recovery, CRISPR/Cas9 RNP electroporation, and immediate embryo transfer within a single caprine reproductive workflow. The combination of morula development with detectable MSTN modification, pregnancy following transfer of electroporated presumptive zygotes, fetal development to 57 days, and detection of target-site

modification in a recovered fetus provides a foundation for further development of MOET-compatible embryo genome-editing strategies in goats.

Conclusion

The present study provides proof-of-concept for CRISPR/Cas9-mediated editing of the caprine MSTN gene using RNP electroporation of *in vivo* derived presumptive zygotes recovered through MOET. The ability of electroporated embryos to progress through *in vitro* development to the morula and blastocyst stages demonstrates the compatibility of the electroporation approach with subsequent embryonic development. Detection of MSTN locus modification in morula-stage embryos and in one of the recovered fetuses further supports the potential of this approach to introduce genome modifications that can persist through subsequent stages of development. Moreover, the successful development of transferred 4-cell embryos to fetuses demonstrates the developmental competence of the edited embryo cohort. Collectively, these findings establish a feasible integrated platform combining MOET, CRISPR/Cas9 RNP electroporation, *in vitro* embryo culture, and embryo transfer for genome editing in goats, providing a foundation for further optimization of editing efficiency and validation of heritable MSTN modifications.

Materials and Methods

Ethical approval

The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the ICAR–Central Institute for Research on Goats, Makhdoom, Mathura, Uttar Pradesh, India (Approval No. IAEC/CIRG/24–25). All experimental procedures involving animals were performed in accordance with the relevant institutional guidelines and regulations and the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study was conducted and reported in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Experimental design

The study was designed to evaluate CRISPR/Cas9-mediated editing of the caprine MSTN gene in *in vivo* derived presumptive zygotes obtained through MOET. A sgRNA targeting exon 1 of MSTN was complexed with recombinant Cas9 protein to form the RNP complex. Presumptive zygotes (n = 78) recovered from eight donor does were electroporated with the RNP complex under optimized conditions. The presumptive zygotes were cultured *in vitro* and developed to the morula and blastocyst stages. Four-cell embryos (n = 4) were transferred to a synchronized recipient doe for assessment of *in vivo* developmental competence, while four morulae were individually analyzed for genome editing. Molecular assessment involved genomic DNA (gDNA) isolation, whole-genome amplification (WGA), PCR amplification, and T7 Endonuclease I (T7E1) assay (Fig. 1).

Designing of MSTN-specific guides targeting exon 1

Exon 1 of the caprine *MSTN* gene was selected as the target region for CRISPR/Cas9-mediated genome editing. A dual-component guide RNA system consisting of a target-specific CRISPR RNA (crRNA) and a constant trans-activating CRISPR RNA (tracrRNA) was used for *MSTN* targeting. The target-specific crRNA was designed against a 20-nucleotide sequence within exon 1 using the CRISPOR web-based design platform (http://crispor.tefor.net/). Candidate target sites were assessed on the basis of their predicted targeting efficiency and off-target potential, and a guide exhibiting a high specificity score with minimal predicted off-target activity was selected for subsequent experiments. The oligonucleotides corresponding to the selected target sequence were commercially synthesized and used for preparation of the guide RNA. The oligonucleotide sequences used in this study are provided in Supplementary Table S1. The guide RNA design strategy was adapted from that described by Punetha et al.[12].

In vitro synthesis and purification of sgRNA

The selected *MSTN* targeting sgRNA was synthesized by *in vitro* transcription (IVT) using the Precision gRNA Synthesis Kit (Catalog #A29377; Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. PCR amplification of the DNA template was performed using a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). All PCR reagents were used according to the manufacturer's recommendations, whereas the thermal cycling conditions were optimized for the present study (Supplementary Table S2). Successful amplification of the target DNA fragment was confirmed by electrophoresis on a 2% agarose gel followed by visualization using a GelDoc Go Gel Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The verified PCR product served as the template for *in vitro* transcription. The sgRNA was synthesized and purified according to the manufacturer's protocol, quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), aliquoted, and stored at −80°C until further use.

Preparation of CRISPR/Cas9 RNP complex

Immediately before electroporation, the CRISPR/Cas9 RNP complex was freshly prepared by combining 2 µL of TrueCut™ HiFi Cas9 Protein v2 (Catalog #A50576; Invitrogen, Thermo Fisher Scientific, USA) with 1.5 µL of purified sgRNA. The mixture was incubated at room temperature for 10 min to facilitate RNP complex formation. Following incubation, the complex was diluted with Opti-MEM™ medium (Catalog #31985062; Thermo Fisher Scientific, USA) to obtain a final volume of 40 µL, which was used as the electroporation solution.

Superovulation of donor goats

A total of eight apparently healthy, adult, primiparous, donor does with body condition score of 4 (in the scale of 5) were selected from the experimental shed of the ICAR–Central Institute for Research on Goats, Makhdoom, Mathura, Uttar Pradesh, India, for the superovulation and collection of *in vivo* derived presumptive zygotes using a standardized MOET protocol. Estrus synchronization, superovulatory

hormone administration, estrus detection, natural breeding, and embryo recovery were performed according to the experimental schedule presented in Supplementary Table S3. Throughout the treatment period, the donor animal was closely monitored, and all hormonal treatments and reproductive procedures were performed under the supervision of experienced veterinarian.

Recovery of presumptive zygotes from donor goats

Presumptive zygotes were recovered from superovulated donor does 20 h after breeding through laparoscopy-assisted embryo collection. Briefly, feed and water were withheld from donor does for 24 h and 12 h, respectively. Sedation was induced by intramuscular administration of ketamine (2 mg/kg body weight) and xylazine (0.1 mg/kg body weight). Following preparation of the surgical site, local anesthesia was achieved by subcutaneous infiltration of 2% lignocaine at the trocar insertion sites. No euthanasia was performed as part of this study.

A two-port laparoscopic technique was employed for embryo recovery. Following establishment of pneumoperitoneum, a laparoscope (0 degree, 5 mm) and atraumatic Babcock forceps (5 mm) were introduced through 5-mm ports to visualize the reproductive tract and ovaries and to assess the superovulatory response. The uterine horn was grasped using atraumatic grasping forceps, and the trocar insertion site was slightly enlarged using a scalpel to exteriorize the uterine horn and ovary. Once exteriorized, the fimbria of the ovary was gently held, and one end of a sterile silicone tubing (1 mm diameter and 10 cm long) was inserted and fixed at the fimbrial end of the oviduct, while the other end of the tube was placed in a pre-warmed (37°C) Petri dish. The uterine horn was then punctured using a blunt 20G needle and gently flushed towards the fimbrial end using pre-warmed (37°C) flushing medium consisting of Dulbecco's phosphate-buffered saline (DPBS) supplemented with calcium and magnesium (Catalog #14040-117; Gibco), bovine serum albumin (1 mg/mL; Catalog #A3059; Sigma-Aldrich, USA), and gentamicin (50 µg/mL; Catalog #G1264; Sigma-Aldrich, USA). The flushed contents were collected through the silicone tubing into the Petri dish. The same procedure was repeated for the contralateral reproductive tract.

Finally, the recovered flushing medium was examined under a stereomicroscope (Nikon SMZ1270) for the recovery of presumptive zygotes. Recovered presumptive zygotes were washed thoroughly in oocyte and embryo wash medium (Vitrogen) to remove residual flushing medium and cellular debris before further processing. **All recovered presumptive zygotes were evaluated for morphological quality using an inverted microscope at higher magnification (Eclipse Ts2R; Nikon Corporation, Tokyo, Japan) and subsequently subjected to CRISPR/Cas9 RNP-mediated electroporation.**

The uterine horns and ovaries were then gently reposed into the abdominal cavity. Upon completion of the procedure, the abdominal incision and laparoscopic port sites were sutured as per the standard procedure followed by intramuscular injections of broad-spectrum anti-microbial, anti-inflammatory and, anti-histaminic drugs along with local dressing of surgical wounds using anti-microbial ointment. The donors were monitored until complete post-operative recovery. On the day 10 post-breeding, an intramuscular injection of 250µg Cloprostenol was administered to donor does so as to regress the

multiple corpora lutea and, to flush out any unrecovered embryo to avoid ectopic or undesired pregnancy.

Electroporation of MOET-derived presumptive zygotes

Immediately before electroporation, all recovered presumptive zygotes were washed at least three times in Opti-MEM™ medium (Catalog #31985062; Thermo Fisher Scientific, USA) to eliminate residual oocyte and embryo wash medium that could interfere with the conductivity of the electroporation solution. The washed presumptive zygotes were transferred into a sterile 1-mm gap electroporation cuvette (Catalog #1652083; Bio-Rad Laboratories, USA) containing 40 µL of the freshly prepared RNP complex.

Electroporation was performed using a Gene Pulser Xcell™ Total System (Bio-Rad Laboratories, Hercules, CA, USA) operating in square-wave mode. Square-wave pulses were selected because they generate a constant electric field during pulse delivery, resulting in transient membrane permeabilization that facilitates efficient intracellular uptake of macromolecules while minimizing irreversible membrane damage. **All recovered MOET-derived presumptive zygotes were electroporated** using three square-wave pulses at 25 V with a pulse duration of 3 msec and an interval of 100 msec between successive pulses.

Post-electroporation recovery and in vitro culture of presumptive zygotes

Following electroporation, the presumptive zygotes were allowed to recover in the electroporation cuvette for 10 min to facilitate restoration of plasma membrane integrity after transient electroporation-induced permeabilization. Subsequently, the presumptive zygotes were gently retrieved by flushing the cuvette two to three times with oocyte and embryo wash medium to maximize embryo recovery. The recovered presumptive zygotes were washed thoroughly and **maintained under standard *in vitro* embryo culture conditions in Vitrogen IVC medium at 37°C in a humidified tri-gas incubator (Cooper Surgical Planer BT37, UK). Embryonic development was monitored morphologically throughout the culture period.**

Molecular confirmation of genome editing in in vitro cultured embryos

gDNA isolation

Four individual morula stage embryos (n=4) were selected for molecular analysis and processed separately for gDNA isolation using the ARCTURUS™ PicoPure™ DNA Extraction Kit (Catalog #1181500; Applied Biosystems™, Thermo Fisher Scientific, USA) according to the manufacturer's instructions with minor modifications. Prior to gDNA extraction, the zona pellucida was removed by treatment with 0.25% Protease (Catalog #P8811; Sigma-Aldrich, USA) to facilitate efficient cell lysis. DNA quality was evaluated by electrophoresis on a 1% agarose gel and visualized using an imaging system. gDNA isolated was stored at −80°C until further analysis.

WGA

Because only a limited quantity of gDNA could be recovered from individual embryos, WGA was performed to generate sufficient DNA for molecular analyses. Amplification was carried out using the Phi29 XT Whole Genome Amplification Kit (Catalog #E1604S; New England Biolabs, USA) according to the manufacturer's instructions. Amplified DNA samples were stored at −80°C until use. The quality of the amplified DNA was assessed by electrophoresis on a 1% agarose gel followed by visualization using an imaging system.

PCR amplification

Amplified DNA obtained from individual embryos served as the template for PCR amplification. PCR amplification was performed using Q5® Hot Start High-Fidelity 2× Master Mix (Catalog #M0494S; New England Biolabs, USA) under optimized reaction conditions. Details of the PCR reaction mixture and thermal cycling conditions are provided in Supplementary Tables S4 and S5, respectively. Amplified PCR products were resolved on a 2% agarose gel and visualized using an imaging system.

T7E1 assay

Genome editing was evaluated using the EnGen® Mutation Detection Kit (Catalog #E3321S; New England Biolabs, USA) according to the manufacturer's instructions. Briefly, PCR amplicons were denatured and re-annealed to allow heteroduplex formation between wild-type and edited DNA strands, followed by digestion with T7 Endonuclease I. The digestion products were separated by electrophoresis on a 2% agarose gel and visualized using an imaging system.

Laparoscopic transfer of electroporated presumptive zygotes into synchronized recipient doe

During *in vitro* culture, four cell embryos (n = 4) derived from the electroporated MOET embryos were randomly selected for embryo transfer. These embryos were laparoscopically transferred into an apparently healthy, adult, multiparous, synchronized recipient Sirohi doe aged 42 months, with 40 kg body weight and body condition score of 4 (on a scale of 5), maintained in the experimental shed of the ICAR–Central Institute for Research on Goats, Makhdoom, Mathura, Uttar Pradesh, India, under strict aseptic conditions. The recipient doe was in natural synchrony of the estrous cycle with that of the donor doe to ensure developmental synchrony between the transferred embryos and the uterine environment.

Prior to surgery, feed and water were withheld for 24 h and 12 h, respectively. Sedation was induced by intramuscular administration of ketamine (2 mg/kg body weight) and xylazine (0.1 mg/kg body weight). Following preparation of the surgical site, local anesthesia was achieved by subcutaneous infiltration of 2% lignocaine at the trocar insertion sites. No euthanasia was performed as part of this study.

A two-port laparoscopic technique was employed for embryo transfer. Following establishment of pneumoperitoneum, a laparoscope (0 degree, 5 mm) and atraumatic Babcock forceps (5 mm) were introduced through 5-mm ports to visualize the reproductive tract and the ovary with an active corpus luteum. **The four cell embryos (n=4) were transferred into the ipsilateral uterine horn corresponding to the ovary bearing an active corpus luteum.** The uterine horn was grasped using atraumatic grasping

forceps, and the trocar insertion site was slightly enlarged using a scalpel to exteriorize the uterine horn and ovary. Once exteriorized, the fimbria of the ovary was gently held and the embryo transfer catheter was introduced through the fimbria towards the uterine end, where the embryos were gently deposited.

The uterine horn and ovary were then gently reposed into the abdominal cavity. Upon completion of the procedure, the abdominal incision and laparoscopic port sites were sutured as per the standard procedure, followed by intramuscular administration of broad-spectrum antimicrobial, anti-inflammatory, and antihistaminic drugs along with local dressing of surgical wounds using antimicrobial ointment. The recipient was monitored until complete post-operative recovery.

Pregnancy diagnosis

Pregnancy was diagnosed on day 29 following embryo transfer by using B-mode real-time transrectal ultrasonography (CHISON®). Successful pregnancy was confirmed by the presence of embryonic vesicles containing viable fetuses with detectable fetal heartbeats.

Recovery of fetuses and gDNA isolation

The recipient doe subsequently died due to pneumonia on day 57 of gestation. A post-mortem examination was performed immediately, and two intact fetuses were recovered aseptically from the uterus. The fetuses were examined for gross morphological abnormalities and fetal developmental status based on their external morphology and morphometric characteristics in accordance with Sivachelvan et al.[13]. Ear pinna tissue was collected separately from each fetus for subsequent gDNA isolation and molecular analyses.

gDNA was isolated independently from each tissue sample using the PureLink™ Genomic DNA Mini Kit (Catalog #K182001; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. gDNA was subsequently purified following the kit protocol and stored at −80°C until further analyses. DNA concentration and purity were determined using a NanoDrop spectrophotometer, while DNA integrity was assessed by electrophoresis on a 1% agarose gel and visualized using an imaging system.

Detection of genome editing in fetal tissues

Genome editing in fetal tissues was evaluated using the EnGen® Mutation Detection Kit according to the manufacturer's instructions. Briefly, PCR amplicons generated from fetal gDNA were denatured, re-annealed to allow heteroduplex formation, and subsequently digested with T7 Endonuclease I. The digestion products were resolved by electrophoresis on a 2% agarose gel and visualized using an imaging system.

Statistical analysis

Descriptive data were generated using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA). Molecular analyses, including PCR amplification and T7 Endonuclease I (T7E1) assay, were interpreted qualitatively based on the presence or absence of the expected amplification products and cleavage fragments, respectively.

Declarations

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Author contributions

SPS: Writing –review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **JP:** Writing – original draft, Methodology, Formal analysis, Data curation. **MP:** Formal analysis, Methodology, Data curation. **AG:** Investigation, Formal analysis. **YKS:** Investigation, Formal analysis. **MKC:** Supervision and Investigation. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this article and its supplementary information files.

Declaration of competing interest

The authors declare no competing interests.

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Figures

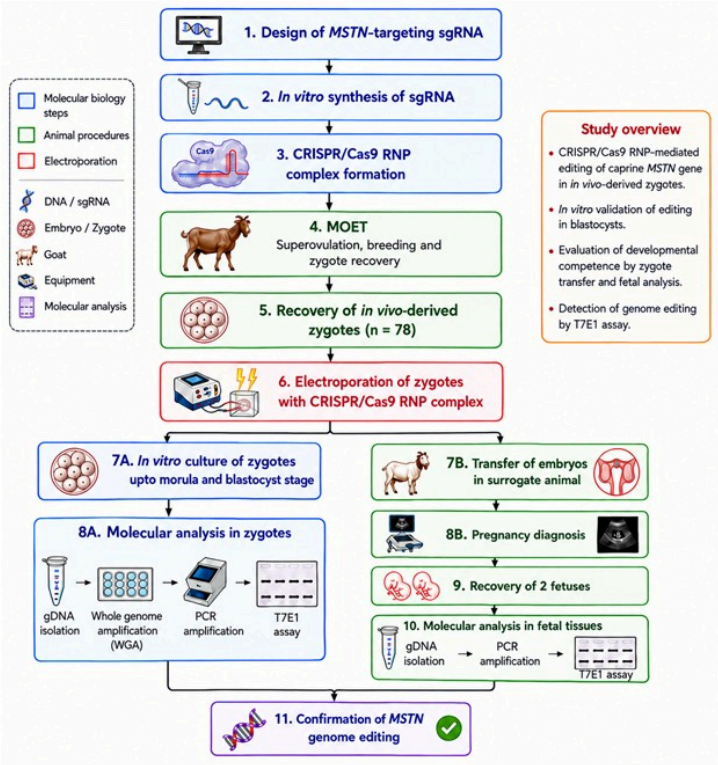


Fig. 1 Experimental workflow illustrating CRISPR/Cas9-mediated MSTN genome editing in MOET-derived caprine embryos.

Figure 1

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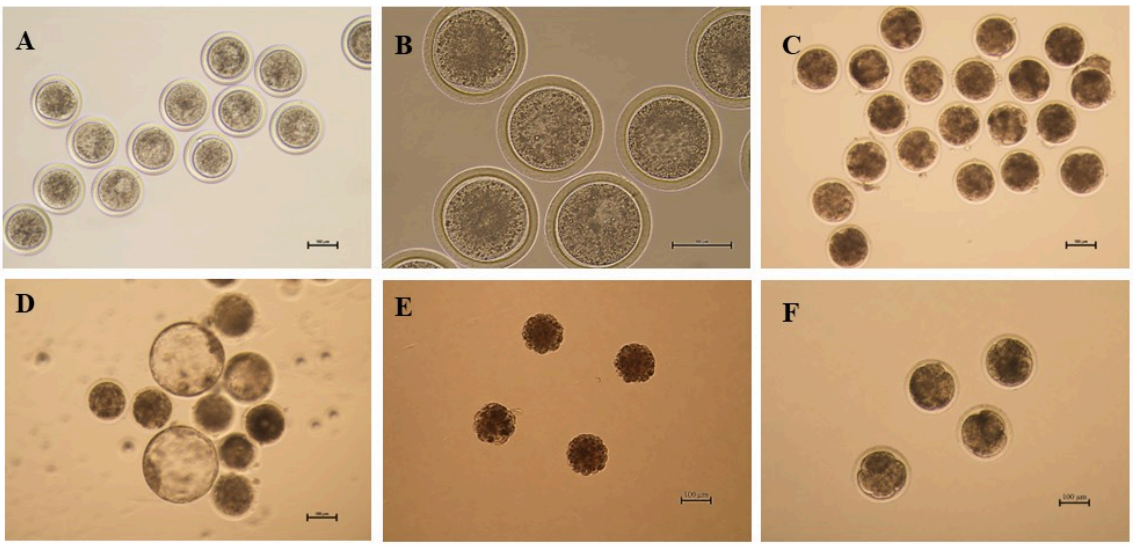


Fig. 2 Morphological assessment and developmental progression of CRISPR/Cas9 RNP-electroporated caprine presumptive zygotes during *in vitro* culture and subsequent selection for molecular analysis and embryo transfer. **A.** Representative image of presumptive zygotes immediately following RNP electroporation. **B.** Enlarged view of electroporated presumptive zygotes exhibiting uniform cytoplasmic appearance and intact zona pellucida. **C.** Representative image of different stages of embryonic development observed during *in vitro* culture of embryos post electroporation. **D.** Embryonic development through the morula stage, with two embryos progressing further to the blastocyst stage. **E.** Morula-stage embryos after enzymatic removal of the zona pellucida with 0.25% pronase for genomic DNA isolation and molecular analysis. **F.** Four 4-cell embryos randomly selected for transfer into a synchronized recipient doe to assess their *in vivo* developmental competence.

Figure 2

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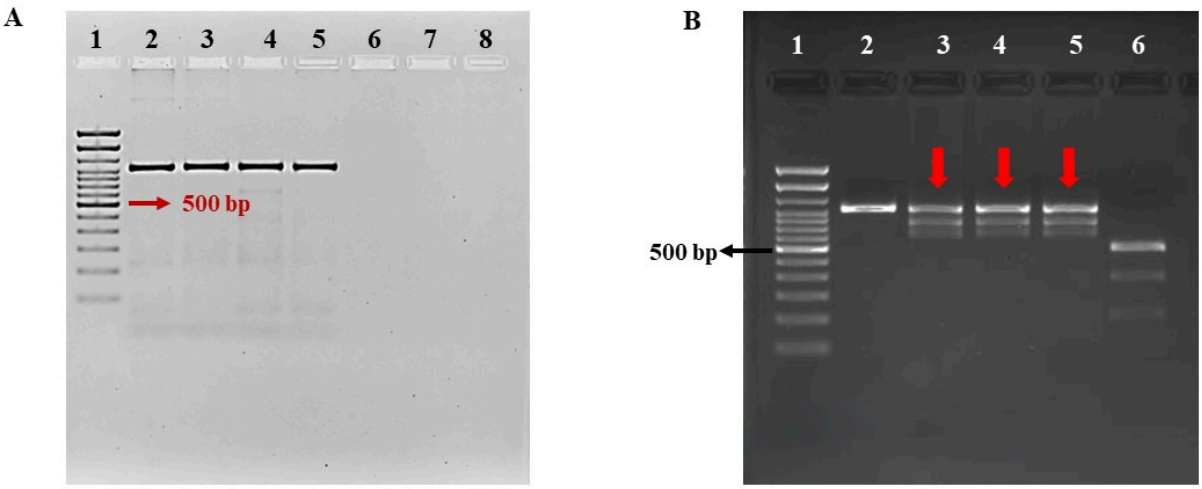


Fig. 3 Molecular confirmation of CRISPR/Cas9-mediated *MSTN* genome editing in electroporated morulae. **A.** Agarose gel electrophoresis showing successful whole genome amplification (WGA) products obtained from genomic DNA isolated from individual morula-stage embryos. **B.** T7 endonuclease I (T7E1) assay demonstrating CRISPR/Cas9-induced mutations in the targeted *MSTN* locus. Cleavage fragments were detected in three of the four morulae analyzed, confirming successful genome editing.

Figure 3

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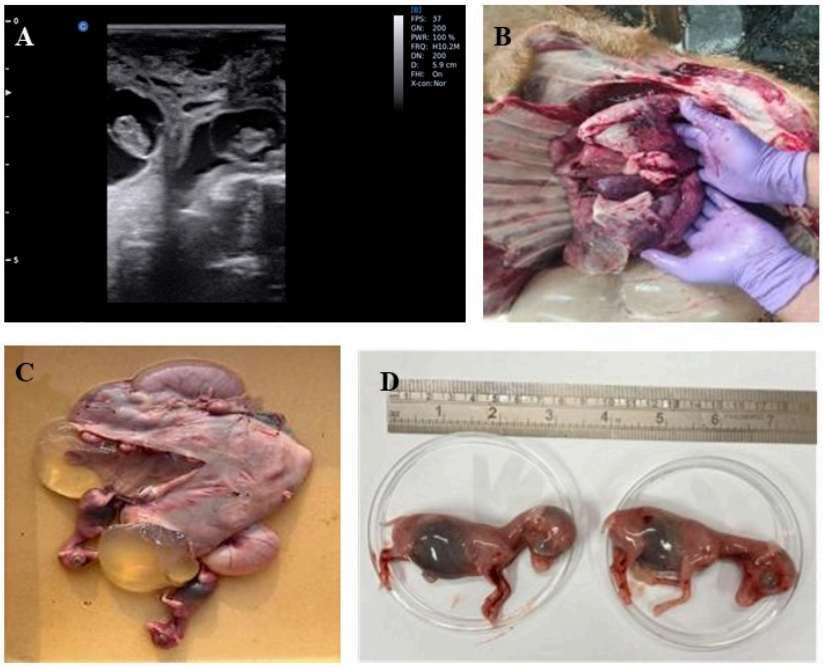


Fig. 4 (A-D) Pregnancy establishment and fetal recovery following electroporation-mediated CRISPR/Cas9 editing. **A.** Transabdominal ultrasonography on day 29 showing a twin pregnancy in the surrogate doe, with two viable fetuses indicated by arrows, confirming successful embryo implantation and pregnancy establishment. **B.** Post-mortem examination of the surrogate doe showing the gravid uterus following death during gestation. **C.** Uterus containing the implanted fetuses recovered during post-mortem examination. **D** Recovered fetuses showing normal gross morphology.

Figure 4

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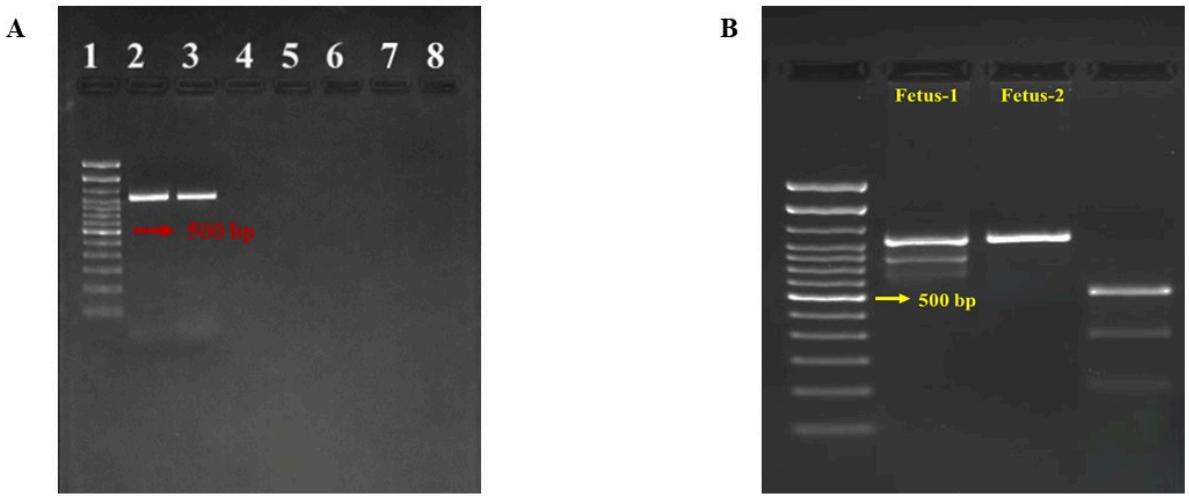


Fig. 5 Molecular analysis of recovered fetuses for detection of CRISPR/Cas9-mediated editing at the MSTN locus. **A.** Agarose gel electrophoresis showing PCR amplification of the targeted MSTN locus from gDNA isolated from ear tissue of the recovered fetuses. **B.** T7 endonuclease I assay of the amplified MSTN locus showing cleavage fragments in Fetus 1, indicative of genomic editing, whereas Fetus 2 showed no detectable cleavage fragments, indicating no detectable editing at the targeted locus.

Figure 5

See image above for figure legend

Supplementary Files

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