Correction of a CFTR/G542X Mutation Using CRISPR/Cas9 Editing in Ovine-bovine Interspecies Embryos

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Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Mutations in this gene result in either a lack of CFTR protein or a defective CFTR protein that cannot perform its key function in the cell. CFTR protein is an anion-selective channel that allows primarily chloride ion but also bicarbonate movement across epithelial cell membranes. Aberrant CFTR thus results in dysregulation of epithelial fluid transport in the lung, pancreas, and other organs. Common pathologies of CF include recurrent lung infection and inflammation, pancreatic insufficiency, which if untreated gives rise to malnutrition, intestinal obstruction, and reduced growth. There are approximately 70,000 individuals living with CF worldwide, with a frequency of 1 in 2,000–3,000 among Caucasian newborns.

To date, over 2000 CFTR mutations have been identified and categorized into 6 types based on their functional effects including protein translation, cellular processing, and channel gating. G542X is a primary mutation in the nonsense mutation category, the second most common mutation type, accounting for 4.6% of CF patients. Nonsense mutations generate a premature stop codon, leading to early termination of CFTR translation, mRNA decay through the surveillance pathway, and ultimately, lack of CFTR function. Some drugs, such as gentamicin (G418) and ataluren (PTC124) has been developed as potential treatments for CF patients with nonsense mutations, which can promote ribosomal readthrough of premature termination codons. However, the use of G418 was limited due to its severe renal toxicity and ototoxicity and ataluren showed no statistically significant difference compared to placebo in phase 3 clinical trials. In addition, it has been proved that inhibiting the nonsense-mediated mRNA decay pathway with antisense oligonucleotides can upregulate target RNA and functional protein expression in cells homozygous for the CFTR/W1282X mutation, which is the second most common nonsense mutation that causes CF. However, no further clinical trials using this strategy have been reported. Therefore, development of a safe and effective treatment for CF with nonsense mutations remains elusive.

Genome editing technologies, especially those based on CRISPR/Cas9, have been successfully applied in genome manipulation in different animals. Recent successes in precise genome editing trials in early-stage or cloned human embryos have suggested a potentially true cure for genetic diseases. Genome editing of human embryos causes huge concerns because of ethical issues and technical uncertainties regarding the efficiency, mosaicism, off-target effect, and unintended homologous recombination. Genome editing by CRISPR/Cas9 generates double-strand breaks (DSBs) that evoke the error-prone non-homologous end joining (NHEJ) DNA repair pathway, which might cause off-target mutagenesis. During the S to G2 phases of cell cycle, DSBs can also activate an alternative DNA repair pathway, the homology-directed repair (HDR), that repairs the DSB sites using a template from the homologous chromosome or an introduced exogenous DNA molecule, leading to the introduction of desired mutations. Presently, CRISPR/Cas9 is predominantly employed to generate gene knockouts through NHEJ repair pathway. Because HDR efficiency is relatively low, applications of HDR strategy for gene therapy, especially in human germline, have been limited. To address the concerns raised by germline editing, especially in humans, extensive experiments should be conducted, thus, increasing the demand for oocytes and embryos, of which the source is rare, and the use strictly regulated in some species.

Interspecies somatic cell nuclear transfer (iSCNT) is a cloning technique that utilizes recipient oocytes and donor cells derived from two different species. The iSCNT experiments primarily focused on applications of preservation/rescue of endangered species and establishment of embryonic stem cell lines for regenerative medicine research. Reprogramming of human somatic cells by iSCNT has been conducted in many labs without generating the ethical issues surrounding the use of human oocytes. It has been proved that iSCNT embryos between two species that are closely related can develop to term and lead to healthy cloned animals. The in vitro development of monolae or blastocysts has been achieved when nuclear donor cells and recipient oocytes had a very distant taxonomical relation, as in the case of interfamily bovine - pig, interorder cat and panda - rabbit, human - rabbit, - cattle, - sheep, and - goat, though remains ineffective and results are not always reproducible. In the present study, we hypothesized that 1) iSCNT could be an alternative approach to produce embryos for testing gene-editing at embryonic stage in certain species, where oocytes are rare or difficult to obtain, such as sheep in this study, and 2) CRISPR/Cas9-mediated HDR could be used for the correction of the G542X mutation in CF. First, we investigated the developmental potential of ovine-bovine iSCNT embryos. Based on the developmental results of reconstructed embryos, we optimized the concentrations of Cas9/gRNA for cytoplasmic injection and developed a strategy that allowed the correction of the CFTR/G542X mutation by CRISPR/Cas9 editing at embryonic stage.
Results

In vitro development of cloned ovine-bovine embryos

The in vitro development results of cloned ovine-bovine embryos and control bovine-bovine embryos is summarized in Table 1. eGFP bovine and ovine fibroblast cells were used as nuclear donors for SCNT. During the SCNT procedure, we observed no significant difference \((P > 0.05)\) in fusion rates (92.3% vs. 81.3%) between the two groups, indicating that our protocol for cloning of bovine embryos was suitable to produce ovine-bovine iSCNT embryos. Moreover, no difference \((P > 0.05)\) was observed in cleavage rates (85.5% vs. 86.3%) between the groups. The in vitro development of iSCNT embryos was arrested at the 16-cell stage and no morulae and blastocysts were observed (Fig. 1); however, 19.5% of embryos in the control group developed normally to blastocyst stage.

| Donor cells | Recipient oocytes | No. of oocytes | No. of fused (%) | No. of cultured | No. of cleaved (%) | No. of blastocysts (%) |
|-------------|-------------------|----------------|-----------------|-----------------|-------------------|------------------------|
| Sheep       | Cattle            | 130            | 120 (92.3) \(^a\) | 110             | 94 (85.5) \(^a\)  | 0 (0) \(^a\)          |
| Cattle      | Cattle            | 128            | 104 (81.3) \(^a\) | 95              | 82 (86.3) \(^a\)  | 16 (19.5) \(^b\)      |

\(^a, b\) Different superscripts in the same columns indicate significant difference \((P < 0.05)\) between two groups. The data were analyzed by \(t\)-test, and the experiments repeated three times.

CRISPR/Cas9-introduced indel mutations in genome of iSCNT embryos

Specific PCR primers (F: ATGGTTGCATTGGAAGGA, R: CCCTGTGCTAGGCGAGAT, 495 bp) were designed and used to amplify exon 12 of sheep CFTR gene and partial intron sequences. Based on the sequencing results, the guide RNA sequence, gRNA_1: AACATAGTTCTTGGAGAAGG, was used to target the G542 locus of wild-type sheep CFTR gene (Fig. 2a). A HpyAV enzyme site was identified in gRNA_1, which could be employed to facilitate indel mutation detection. Three days after injection of different concentrations of Cas9:gRNA_1 RNP, mutations in target loci were analyzed in each of iSCNT embryos using PCR/RFLP assays and Sanger sequencing (Fig. 2b and 2c). The mutation efficiency reduced to 54.5% when the concentration decreased to 5.3 ng/µl:0.14 µM and no biallelic mutations were found using 1.3 ng/µl:36 nM of Cas9:gRNA_1 RNP for injection. Mosaicism in iSCNT embryos was detected when lower concentrations of RNP (64- to 1024-fold dilution) were used (Supplementary Fig. 1). The cleavage rates of injected iSCNT embryos varied from 77.8 to 100% and no significant difference \((P > 0.05)\) was observed among different concentration groups.

Correction of the CFTR/G542X mutation in genome of iSCNT embryos

The G542X fetal fibroblasts were isolated from a cloned G542X fetus produced in our laboratory (unpublished) and subsequently used as nuclear donor cells for the establishment of cloned ovine-bovine embryos. We genotyped G542X fibroblasts by PCR amplification and Sanger sequencing. The results verified the presence of homozygous G542X mutations in this cell line, in which the first nucleotide ‘G’ at G542 site of CFTR was replaced by a ‘T’, resulting in the formation of a stop codon ‘TGA’ (Fig. 3a and 3c). Based on the sequencing results, a gRNA_2: AACATAGTTCTTGGAGAAGG, and a ssODN, 5’-T*T*TGATAATAGCAATCTCAAGTTTTCAGAGAAAGACACATAGTTCTTGGAGAAGGTCACATTGAGTTGAGGTCACCGAGCAAGAGAATTCTTCTT*T*A-3’, were utilized to correct the G542X mutation in the iSCNT embryos (Fig. 3a). After injection of Cas9/gRNA_2 and ssODN compound, the correction of the G542X mutation in injected embryos was confirmed by using PCR and Sanger sequencing (Fig. 3b and 3c). When 1.4 µg/µl:36 µM:73 µM and 0.09 µg/µl:2.3 µM:18 µM of Cas9:gRNA_2:ssODN compound were used for injection, we obtained a correction rate of 5.7% (3/53) and 11.1% (1/9), respectively (Fig. 3d). No correction was observed (0/15) when 0.09 µg/µl:2.3 µM:4.5 µM ssODN was used. The cleavage rates varied from 89.0% to 90.5% and no significant difference \((P > 0.05)\) was observed among these three groups.

Off-target (OT) detection of mutated and corrected iSCNT embryos

We chose 11 potential OT sites in sheep genome related to the gRNA_1 and 6 OT sites for the gRNA_2 with the highest sequence homology (scores > 1) (Supplementary Table 1). Specific PCR primers were designed to amplify DNA fragments ranging from 300 bp to 900 bp spanning each potential OT locus (Supplementary Table 2). Genomic DNAs derived from whole genome amplifications of 5 mutated and 4 G542X-corrected iSCNT embryos were used as the templates for PCR amplification. Sequencing results of PCR products verified that none of these embryos had mutations in the analyzed OT sites (Supplementary Fig. 2).
Discussion

The generation of interspecies embryos by cloning, reported more than 30 years ago, uncovered an distinctive fact that they could be the most useful applications for researching the mechanism of nuclear-cytoplasm interactions and conservation and rescue of endangered or extinct species. Here, we expand its applications to the evaluation of embryo gene-editing in species where oocytes are scarce or an access to them is limited. For instance, our lab has a limited access to sheep oocytes while we can routinely obtain a large quantity of cattle oocytes. Our data indicate that ovine-bovine interspecies embryos can be effectively gene-edited by CRISPR/Cas9 approach.

We generated ovine-bovine cloned embryos using eGFP ovine fibroblasts as nuclear donors, which allowed us to accurately differentiate cloned embryos from those parthenogenetically activated and evaluate the development capacity of reconstructed embryos. The ovine-bovine embryos successfully developed to 16-cell stage but arrested prior to the formation of morulae or blastocysts. Our results are consistent with those published by Lagutina et al., who reported the development of iSCNT embryos to 12- to 16-cell stage. Hua et al. (2008) observed 24.6% of blastocyst development with 117 ± 13 cells per blastocyst from such iSCNT embryos. It has been reported in many studies that the bovine oocytes as recipient cytoplasm support the development at least to 10- to 16-cell of iSCNT embryos produced using nuclear donor cells from other species, including buffalo, sheep, mouse, cat, rabbit, horse. The morula or blastocyst development of iSCNT embryos has been observed even when nuclear donor cells have a distant taxonomical relationship with bovine recipient oocytes, as in the case of interclass chicken-cow, interorder pig-, dog-, monkey- and human-cow. The gaur-bovine iSCNT embryos were able to implant and develop to term. Though term development of iSCNT embryos only limited in several closely related species, short-term in vitro developmental capacity of these embryos can provide an efficient window stage and enough genome materials for gene and mutation analysis.

Injection of Cas9/gRNA compound has been shown to be effective for gene editing of embryos in large animals. Assessing mutation efficiency of Cas9/gRNA compound in embryos prior to embryo transfer is critical because it is a time-saving and cost-effective strategy to generate CRISPR-mutated large animals. Our results showed that the bases encoding G542 amino acid of sheep CFTR gene in genome of ovine-bovine iSCNT embryos can be disrupted with a high efficiency of 88.9–100% through injection of Cas9/gRNA RNP at different concentrations. Typically, higher RNP concentrations is more efficient, while also lead to increased embryonic toxicity. However, we observed no difference in embryo cleavage rates when different concentrations of RNP were used, thus, a wide concentration range is available to test and optimize reagents for efficient gene editing in embryos.

An important factor to be considered in gene editing of embryos using the RNP injection approach is the occurrence of mosaicism in edited embryos, which results from indel introduction after the first round of DNA replication. We observed embryos with mosaic mutations when lower concentrations of RNP were used. Our results are consistent with findings in porcine zygotes editing experiments, which reported that lowering Cas9 protein concentrations from 100 ng/µl to 20 ng/µl for cytoplasmic injection decreased both mutation efficiency and the rates for biallelic mutations. The minor discrepancy between our and their results is that we achieved higher biallelic mutation efficiency when using 20 ng/µl of Cas9 protein for injection compared to no biallelic mutations in analyzed porcine blastocysts in their study. Differences in embryos, genes, reagents, and injection methods may account for this discrepancy. Different concentrations of RNP used for embryonic injection in large animals has been reported in other experiments with variable outcomes. Therefore, we suggest that it is necessary to optimize injection conditions before starting to generate embryonic cells for transfer.

The G542X mutation is the most common nonsense mutation in CFTR. Currently, there are no available treatments for CF nonsense mutations that are both safe and clinically effective. In this study, we achieved up to 11.1% of biallelic correction efficiency in ovine-bovine embryos when Cas9/gRNA was co-injected with a 101nt donor ssODN. Using similar strategy and size of ssODN, Miao et al. conducted the correction of a missense mutation, Fgfr3/G374R and achieved 22.5% correction in newborn mice. In our study, we first utilized CRISPR editing to correct a CFTR mutation in ovine-bovine embryos. Our results support the hypothesis that precise genome editing in embryos by CRISPR could be a true cure for certain genetic diseases. The high degree of sequence similarity between farm animals and humans should allow for accurate modeling of single nucleotide correction in human embryos compared to other trials in mouse embryos or in transfected cell lines. In addition, it is noticeable that we did not detect mutagenesis induced by off-targeting in these mutated and corrected embryos, increasing the odds to utilize this strategy for clinical applications.

In conclusion, cytoplasmic injection of Cas9/gRNA RNP results in highly efficient mutation induction in genome of ovine-bovine iSCNT embryos. The CFTR/G542X mutation can be corrected via co-injection of CRISPR/Cas9 RNP and donor ssODN compound into iSCNT embryos, which could be a potential strategy to correct genetic defect in genome at embryo stage. The use of iSCNT embryos for gene editing overcomes the limitation of oocyte source and provides the opportunity of mimicking the editing of any other genes in embryos of different species. Despite the limited developmental competency, the ovine-bovine iSCNT embryos were able to progress to the 16-cell stage that is sufficient for gene-editing optimization and assessment of its efficiency.

Methods

gRNAs and ssODN Design.
Specific primers were designed according to the sheep CFTR genome sequences (GenBank, NC_019461.2) and used to amplify the exon 12 of CFTR and parts of introns flanking it. Based on the sequencing results, we designed gRNAs by searching for the (N)20NGG motifs using the Benchling software (https://benchling.com/academic). gRNA_1 was chosen to induce DSB at G542 locus in exon 12 of the CFTR gene for mutation efficiency detection. A restriction enzyme site was identified in gRNA_1 to facilitate indel mutation efficiency detection using PCR/Restriction Fragment Length Polymorphism (RFLP) assays. gRNA_2 and ssODN were designed to correct the G542X mutation of the CFTR in iSCNT embryos, which were generated by using G542X sheep fetal fibroblasts as nuclear donors. The size of ssODN was 101nt and the mutated nucleotide, 'G', located in the middle of it was flanked with 50 nucleotides at each side. The gRNAs were synthesized by Synthego with chemical modifications (2'-O-methyl 3'-phosphorothioate modifications in the first and last 3 nucleotides). The TrueCut™ Cas9 protein V2 was purchased from Thermo Fisher Scientific. The ssODN was synthesized as the Ultrimer→ DNA oligonucleotides with chemical modifications by Integrated DNA Technologies.

SCNT.

Both bovine SCNT and ovine-bovine interspecies SCNT were performed as described by Fan et al. for goats 34, with modifications wherein an aspiration technique was used for oocyte recovery instead of a slicing technique and bovine oocyte maturation medium instead of caprine medium. The bovine oocyte maturation medium consists of TCM-199 supplemented with 10% fetal bovine serum (FBS, SH30070.03, HyClone) 5 µg/ml luteinizing hormone (L9773, Sigma-Aldrich), 0.5 µg/ml follicle stimulating hormone (F2293, Sigma-Aldrich), and 100 U/ml penicillin/streptomycin (15140-122, Gibco). The bovine and ovine fibroblasts expressing the enhanced green fluorescent protein (eGFP) were constructed and cryopreserved in our lab. The CFTR/G542X sheep fetal fibroblasts were isolated from a cloned fetus collected on day 45 of gestation and cryopreserved in our lab. The fibroblasts at passage 2 were used for second-generation cloning. Both bovine and ovine fibroblasts were grown to 80% ~ 90% confluence and used as nuclear donor cells for SCNT after 24 hours of serum starvation (0.5% FBS in DMEM). The cloned embryos were cultured in caprine SOF medium for 7 days 34. The in vitro development of cloned embryos was observed under a stereo microscope and eGFP expression photographed by a fluorescent microscope (Observer Z1, Zeiss).

Cytoplasmic Injection of iSCNT Embryos.

The iSCNT embryos, collected 4 h after activation, were subjected to cytoplasmic injection. Prior to injection, 2 µl of 100 µM gRNA was incubated with 1.5 µl of 5 µg/µl Cas9 protein for 10 min at room temperature to form a ribonucleoprotein (RNP) complex. The RNP was then incubated with 2 µl of H2O (for KO) or 200 µM ssODN (for KI) for 5 min and the resulting mixture was used for cytoplasmic injection. A group of 30–50 embryos were transferred to a 50 µl of HSOF drop covered with mineral oil 34 and 5–10 pl of Cas9:gRNA or Cas9:gRNA:ssODN compound was injected into the cytoplasm of an embryo. The inter diameter of an injection pipette was 5–7 µm and the injection for each group of embryos was completed within 20 min using a Piezo micromanipulator (PiezoDrill, Burleigh). The injected embryos were temporarily cultured in SOF for 10 min to assess post-injecting survival rate, and then the survival embryos were transferred to a new 30 µl SOF drop for culture.

Mutation analysis of injected embryos.

The embryos were collected 3 days after cloning and injection procedure for mutation detection. Crude DNA from each embryo was prepared according to the method described by Sakurai et al. with some modifications 35. Briefly, under a stereo microscope, 1–2 µl of SOF medium containing 1 embryo was transferred to 10 µl embryo lysis buffer (200 µg/ml proteinase K, 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.02% gelation, 0.45% Tween 20) in a 0.2 ml PCR tube. The mixture was vortexed for 5 s and then incubated at 56 °C for 1 h followed by 95 °C for 10 min in a PCR machine. The resulting crude DNA solution was stored at -20 °C until use. The whole genome amplification was conducted using a REPLI-g Mini Kit (Cat. 150023, Qiagen) according to the manufacturer’s protocol. The products from whole genome amplification of embryos were used for mutation analysis and off-target detection. The mutations of embryos were detected with PCR/RFLP assays and Sanger sequencing as described elsewhere 36. The number of embryos with bi-allelic mutations was calculated and used for indel mutation and correction efficiency analysis.

Off-target analysis.

We conducted a BLAST search of the sheep nucleotide sequence database with two gRNA sequences as the queries to find the genomic sequences with the highest homology using Crispor online software (Version 4.97). We chose potential off-target (OT) sites with high sequence homology (scores above 1) to the seed sequences. Specific PCR primers were designed to amplify DNA fragments of approximate 500 bp spanning each potential OT locus. PCR amplification and Sanger sequencing were used for the OT analysis of the genomic DNAs derived from whole genome amplifications of mutated and G542X-corrected iSCNT embryos.

Statistical analysis.
The experiments for the generation of bovine-bovine and ovine-bovine cloned embryos were repeated for three times. The data for fusion and in vitro development experiments were subjected to arcsine square root transformation and analyzed by unpaired t test. The data for cleavage rates collected during the production of mutated and G542X-corrected embryos were analyzed by x² test. A difference was considered significant when the P value was less than 0.05.

Declarations

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Author Contributions

Z.F., I.A.P., A.H., and K.L.W. participated in study design. Z.F., Y.L., and I.V.P. performed the experiments and analyzed the data. Z.F. and I.A.P. wrote the manuscript. I.A.P., A.H., and K.L.W. supervised the study. All authors discussed the data and reviewed the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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**Figures**
In vitro development of cloned bovine-bovine and ovine-bovine embryos. Cleaved embryos and weak eGFP expression were observed in both groups on Day 2 after activation. The in vitro development of iSCNT embryos was arrested at the 16-cell stage and eGFP expression was weaker in ovine-bovine group than in the control on Day 4. The embryos at morula and blastocyst stages with strong eGFP expression were found in the control group, but not in iSCNT group, when observed on Day 6 and 7. WL, white light.

**Figure 1**

|                      | Bovine - bovine | Ovine - bovine |
|----------------------|-----------------|----------------|
|                      | WL              | GFP            | WL              | GFP            |
| Day 2                |                 |                |                 |                |
|                      | ![Day 2 images](image1) | ![Day 2 images](image2) |
| Day 4                |                 |                |                 |                |
| ![Day 4 images](image3) | ![Day 4 images](image4) |
| Day 6                |                 |                |                 |                |
| ![Day 6 images](image5) | ![Day 6 images](image6) |
| Day 7                |                 |                |                 |                |
| ![Day 7 images](image7) | ![Day 7 images](image8) |

**a** CFTR/Wt

| Wt | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA |
|----|-----------------------------------|
| 11 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 8nt del |
| 12 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 3nt del |
| 13 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 6nt del, 1nt rep |
| 14 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 7nt del |
| 15 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 10nt del |
| 16 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 12nt del |
| 17 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 18nt del |
| 18 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 9nt del |
| 18 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 9nt del |
| 19 | GAGAAAGACAACATAGTTTGTGAGCTGGCACTACA 9nt del |

**b** PCR/RFLP

| Control | Injected | Wt |
|---------|----------|----|
| ![PCR/RFLP image](image9) | ![PCR/RFLP image](image10) | ![PCR/RFLP image](image11) |

**c**

| # | Mutation | Cleavage (%) | Percentage |
|---|----------|--------------|------------|
| 1x | 0 | 100 | 100 |
| 4x | 50 | 50 | 50 |
| 16x | 75 | 75 | 75 |
| 64x | 100 | 100 | 100 |
| 256x | 95 | 95 | 95 |
| 1,024x | 90 | 90 | 90 |

**d** Dilution of Cas9/gRNA

| Dilution of Cas9/gRNA | Percentage |
|------------------------|------------|
| 1x | 100 |
| 4x | 50 |
| 16x | 75 |
| 64x | 100 |
| 256x | 95 |
| 1,024x | 90 |
CRISPR/Cas9-induced indel mutations in sheep genome of iSCNT embryos. (a) Schematic diagram of the target site in sheep CFTR gene. The gRNA sequences flanking the CFTR/G542 locus are depicted, with a HpyAV site used for PCR/restriction fragment length polymorphism (RFLP) assay underlined. Letters in red indicate the protospacer-adjacent motif (PAM). The letter in brown indicates the first nucleotide of the codon for Glycine542 (G542). In the G542X mutation of CFTR gene in humans, the nucleotide ‘G’ is replaced by a ‘T’, resulting in the generation of a stop codon ‘TGA’. Arrows indicate locations of PCR primers. (b) Representative gel electrophoresis results for mutation analysis of ovine-bovine embryos generated by interspecies somatic cell nuclear transfer (iSCNT) and injected with Cas9/gRNA_1 compound. The mutations in target sites in each of injected embryos were detected by PCR/RFLP assays. M, 1-kb DNA ladder; C1-C3, iSCNT embryos in the control group without injection; I1-I3, iSCNT embryos in the experimental group injected with Cas9:gRNA_1 compound. The targeted alleles lost restriction sites through error-prone non-homologous end joining (NHEJ) following Cas9-mediated double-stranded DNA breaks. (c) Representative results for sequencing analysis of mutated iSCNT embryos injected with the Cas9/gRNA_1 compound. The embryos were derived from a group injected with the Cas9/gRNA_1 compound at the highest concentration and biallelic mutations at target sites were observed in each of them. Letters in yellow indicate nucleotides inserted at cleavage sites. (d) Optimization of Cas9:gRNA_1 compound concentrations for injection of iSCNT embryos. High biallelic mutation efficiencies ranging from 88.9-100% were observed after injection of Cas9: gRNA compound at 1- to 64-fold dilutions. The cleavage rates for embryos in different concentration groups varied from 77.8 to 100% and no significant difference (P > 0.05) was found among them. The concentration of 1x Cas9:gRNA_1 is 1.4 µg/µl:36 µM.