

«Usage a CRISPR/Cas9-based for obtaining knockouts of economically significant cattle genes»

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Abstract. An experimental work dealing with the gene modification using the Cas9 RNA-based editing system was performed. Point site-specific breakpoints in gDNA were introduced at the zygote stage by microinjection of spCas9 mRNA protein and guide RNAs into the zygote cytoplasm. Oocytes that extruded the first and second polar bodies were used for the injection. 2 series of microinjections of gene editing designs for early bovine embryos were made. The degeneration ranged from 10% to 56% in different groups. A total of 100 injections were performed. Cleavage was started by 78% of the surviving oocytes; 5 embryos reached the blastocyst stage, which was 16% of the number of dividing embryos. All the resulting embryos were analyzed to evaluate the efficiency of editing. gDNA was isolated from all embryos that had reached the blastocyst stage. Using Sanger sequencing of genes of interest in pre-implantation bovine embryos and biopsies from them, it was shown that in 5 out of 17 embryos resulting from microinjections of guide RNA against the BLG gene and SpCas9 mRNA, and in 2 out of 9 embryos after microinjections of guide RNA against CD209 gene and SpCas9 mRNA, the required genome modifications were found. This is indicative of the high efficiency of this delivery method of the editing system.

1 Introduction

CRISPR/Cas9-based genome editing systems are the universally recognized gold standard for introducing point mutations into mammalian genes of interest to obtain knockouts or expression of mutant protein forms. CRISPR/Cas9 systems are widely used primarily because they are a relatively easy, cost-effective and efficient alternative to traditional genome editing techniques such as ZFN or TALEN. CRISPR/Cas9 system vectors are commercially-available and can be used to simultaneously knock out multiple genes [1]. Many laboratory animal models have been bred using this technique [2, 3]. Meanwhile, while working with small laboratory animals, researchers have an almost unlimited choice of

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targets for editing and studying the consequences of genome editing. Nevertheless, when working with large livestock, the opportunity to study the effects of genome editing is limited due to the fact that the total period of prenatal development, the onset of reproductive age, and subsequent pregnancy in cattle exceeds 2 years.

CRISPR/Cas9 technology may be effectively used to modify the embryonic genome at the zygote stage [4]. There are several options for delivering the CRISPR/Cas9 genome editing system to the zygote by microinjections. The simplest is microinjection of a plasmid with the Cas9 gene and a planned sequence of RNA species. If this method of delivery is used, microinjections are performed into the pronucleus of the zygote. Two other techniques: the delivery of RNA or Cas9 protein and the delivery of RNA involve microinjections into the zygote cytoplasm [5]. Moreover, it is shown that the greatest editing efficiency may be reached by using genetic constructs based on protein or Cas9 RNA. Since the presence of lipid granules in bovine oocytes complicates the pronucleus visualization, the use of RNA-based Cas9 editing systems is preferable not only because of better efficiency, but also because of the needlessness of injecting DNA-based editing systems into the zygote cytoplasm.

While choosing a gene for knockout when working with livestock, several factors must be considered: gene knockout should improve the economic properties of the animal breed, for example, affect disease resistance, improve productivity, change the milk composition, etc. Simultaneously, knockout of the selected gene should not result in embryonic and postnatal mortality; it should not worsen the viability of the animal and its breeding potential. Current genome editing technologies, when applied to cattle, can help those with lactose intolerance or other components of milk. Several attempts have also been made to get genetically modified pathogen-resistant cattle. The deletion of the CD163 receptor obtained using CRISPR resulted in pig resistance to the porcine reproductive and respiratory syndrome virus (PRRSV). [6]. Due to the increased expression of NRAMP1 by CRISPR [7] and increased expression of SP110 by TALEN [8], the resistance to tuberculosis in cattle was improved. One more example is pasteurellosis. It is another respiratory disease of cattle caused by *P. hameolytica*, which secretes leukotoxins having cytotoxic effect and binding to the signal peptide of CD18 proteins on the surface of leukocytes. Studies have proven that ZFN can be used to add a single amino acid to the bovine CD18 protein, which will give genetically modified cattle resistance to *P. hameolytica* [9].

Two genes were selected as targets for editing in this study: *CD209* gene, one of the receptors of the immune system responsible for the penetration of many pathogens into the cell, including, probably, the bovine leukemia virus. In this regard, it seems to be a promising target for genetic modification, which can result in the disease resistance. The second target is a gene encoding milk beta-lactoglobulin, an allergenic protein whose occurrence is undesirable in raw milk used for the production of baby and dietary nutrition.

2 Materials and methods

2.1 Oocyte collection

After slaughtering, the ovaries of cattle were selected and transported to the laboratory at 38.5 C in a controlled temperature for 4-5 hours. Immediately following ovarian extraction, aspiration of visualized follicles from 2 to 15 mm was done by 18G needle attached to a 5-10 ml syringe. Aspiration of follicles and all further work with eggs and embryos was performed in aseptically conditions of the clean zone in laminar flow units with a heated surface of up to 38.5 C.

2.2 In vitro maturation

Maturation of oocytes was performed for 24 hours in BO-IVM (IVF-Bioscience) media coated with mineral oil for tissue cultures (Sage), at a temperature of 38.5 C, a carbon dioxide level of 6.5%, and oxygen – 5.0%.

2.3 Sperm preparation

Cryopreserved bull sperm frozen in 0.5 ml straws were used for in vitro fertilization. The straws were thawed in a water bath with a temperature of 37 °C for 30 seconds. The treatment of 400g of sperm was done by density gradient centrifugation: 3 ml of 80% Percoll (Irvine Scientific) for 15 minutes at room temperature. The sperm precipitate after centrifugation was washed with a buffer medium containing 3 IU of heparin for 10 minutes at 200g.

2.4 In vitro fertilization

After centrifugation and washing, sperm was introduced into the BO-IVF (IVF-Bioscience) media with mature cumulus–oocyte complexes at a concentration of 1.0–2.0 x10⁶ of active sperm in 1 ml. The complexes were completely cleared of cumulus cells and sperm 18 hours after insemination. The occurrence of polar bodies was assessed in the obtained oocytes.

2.5 Freeze/thawing oocytes

Cryopreservation was performed by vitrification using Vitrification Media Kit (Kitazato, Japan) and Cryotop straws (Kitazato, Japan) according to the manufacturer's manual. Vitrified zygotes were stored in liquid nitrogen at a temperature of 196°C below zero. Thawing was done by using a set of Thawing Media (Kitazato, Japan) according to the manufacturer's manual. Thawed zygotes were incubated for 2h in BO-IVT (IVF-Bioscience) media for recovery before injection.

2.6 mRNA preparation

To receive a knockout for the selected genes, microinjection into the cytoplasm of a mixture of SpCas9 mRNA protein and guide RNAs was chosen. All further activities were performed on that basis.

The selection of guide RNAs was done using the CRISPOR online algorithm focused on the search for guides with the least number of non-target sites, the absence of self-complementary sites, and a high MIT index (specificity). To knock out the *CD209* gene, guide RNAs were matched to the second exon of this gene. Only one of the five variants of guide RNAs proposed by the CRISPOR algorithm was chosen to best meet the experimental conditions [10].

To knock out the *BLG* gene, it was decided to introduce a mutation into the promoter of this gene, since it was not possible to select a high-quality guide RNA for the gene itself. Only one of the five variants of guide RNAs proposed by the CRISPOR algorithm for the *BLG* gene promoter was selected to best meet the experimental conditions [11].

To develop guide RNAs (sgRNAs) *in vitro*, a plasmid was used in which the guide RNA sequence is controlled by the T7 promoter.

The cloning of guide RNAs was performed in the pBluescript SK vector (Addgene vector database id 195), in which the core sequence of guide RNAs for SpCas9 was previously cloned using the XbaI and AscI sites with the ability to clone the recognizing sequence of guide RNA from two BbsI sites. To clone, the vector was cut using BbsI-HF restriction

enzyme (New England Biolabs), dephosphorylation of the vector using FastAP enzyme (ThermoFisher Scientific), then purification in agarose gel and subsequent isolation using the Monarch DNA Gel Extraction Kit.

5' – TAGGNNNNNNNNNNNNNNNNNNNN – 3'

3' – NNNNNNNNNNNNNNNNNNNNNCAA – 5'

A scheme for the synthesis of oligonucleotides for cloning guide RNA. Nucleotides were added to complement the cohesive ends formed when the vector was cut by BbsI-HF restriction enzyme.

Cd209-T7-f TAGGCTCGACCACTACACAGTTTG

Cd209-T7-r AAACCAAAGTGTAGTGGTCGAG

BLG-T7-f TAGGATTCGGGGAACCGCGTGGC

BLG-T7-r AAACGCCACGCGGTTCCCCGAAT

Oligonucleotides were phosphorylated using the RNA kinase enzyme (Thermo fisher Scientific), reannealed with each other and ligated with a previously prepared vector and using the T4 DNA ligase enzyme (ThermoFisher Scientific). The ligase mixture was transformed into competent XL1 blue strain cells (Eurogene). The colonies were screened by Isogen PCR kits and pSK T7 rev primers (5'-ttgtatgctcgtcagg-3') and a forward primer used for cloning. Selected into two colonies containing sgRNA were grown in an overnight culture and isolated using the Monarch Plasmid Miniprep Kit. The analysis of the obtained plasmids was performed by Sanger sequencing. The sequencing of the obtained plasmids was done using the pSK T7 rev primer.

The plasmid section containing the T7 promoter and the guide RNA sequence was amplified by polymerase chain reaction (PCR) with primers M13-forv (5'-gtaaacgacggccagt-3') and gRNA-core-rev (5'-gcaaaaaagcaccgactc-3'). The resulting PCR product with a size of about 150 base pairs was reprecipitated by the sequential addition of 1 volume of isopropyl alcohol and 1/10 volume of 3M sodium acetate, and 1 volume of ethyl alcohol, dissolved in water. The RNA synthesis reaction was performed with a commercial MEGAshortscript™ T7 Kit (Thermo Fisher Scientific). 1 mcg of purified PCR product was taken into the reaction mixture and incubated with the components of the kit according to the manufacturer's manual. After stopping the synthesis reaction, TRI Reagent® (MERCK) and bromochlorophenol were added to the RNA; the aqueous phase was transferred to a new test tube; RNA was precipitated with the addition of isopropyl alcohol; the precipitate was washed with ethyl alcohol and dissolved in water. The concentration of the isolated RNA was measured with a spectrophotometer.

To obtain cas9 mRNA restrictase, a commercial HiScribe™ T7 ARCA mRNA Kit (New England Biolabs) was used, which allows to produce capped and polyadenylated RNA, and the pET28a/Cas9-Cys plasmid (addgene#53261). The site containing the open Cas9 reading frame under the control of the T7 promoter was amplified with primers T7-f (5'-TAATACGACTCACTATAGGG-3') and T7 term (5'-GCTAGTTATTGCTCAGCGG-3'). 1 mcg of purified PCR product was taken into the synthesis reaction and incubated with the components of the kit according to the manufacturer's manual. After the reaction was completed, the RNA was purified according to the procedure described above.

A mix was prepared for injection into embryos, focusing on the protocol used in the laboratory. It has showed a high efficiency of making incisions and modifications. The final concentration in 10 ml of the injection mix was 50 ng/ml for cas9 mRNA, CD209 guide RNA or Blg - 25 ng/ml. The finished mixes were stored at a temperature of -80°C; transportation was done on dry ice.

Sanger sequencing was used to search for incisions in the genes of interest in blastocysts. Table 1 shows the sequences of primers for amplification of a genomic fragment with possible incisions.

Table 1. The sequences of primers for amplification of a genomic fragment of *BLG* and *CD209* genes.

Code	Sequence 5'->3'	Fragment length
BLG-F2	CCCCTTCTGGGCGTA	366 bps
BLG-R	GCACCTCGAACCTTCTGGA	
CD209-F	TGAACACAAGGAGCCAGATGAC	429 bps
CD209-R2	GAAGAAGCCCAGTGAGACGA	

The fragment was amplified with a Phire Tissue Direct PCR Master Mix kit using the manufacturer's protocol (Thermo Scientific). The composition of the reaction mixture included the following components: 2X Phire Tissue Direct PCR Master Mix - 10 µl; Primer Forward 10 µM - 1 µl; Primer Reverse 10 µM - 1 µl; DNA - 2 µl; ster. H₂O - 6 µl.

Amplification protocol:

1. 98°C - 5 min;
2. 98°C - 5 sec;
3. 63°C - 5 sec;
4. 72° - 20 sec;
5. GOTO (2) x39;
6. 72° - 1 min.

2.7 Injection of mRNA into the oocyte cytoplasm

The injection was performed on a Narishige micromanipulation unit (Japan) complete with an inverted Nikon microscope. The zygotes were placed in the G-MOPS Plus buffer at the injection (Vitrolife, Sweden). 2 series of microinjections of gene editing designs of early bovine embryos were made.

2.8 Embryo culture

After injection, the embryos were transferred to a BO-IVC (IVF-Bioscience) culture media coated with mineral oil for tissue cultures (Sage). The embryos were cultured at a temperature of 38.5 C, a carbon dioxide level of 6.5%, and oxygen – 5.0% without changing the media for the entire gestation time up to the blastocyst stage.

3 Results

During the series of microinjections of genome editing constructs of early bovine embryos, 5 embryos from all experimental embryos of the blastocyst stage reached (16%). Incisions were found in 5 of 17 embryos after microinjections of guide RNA against *BLG* gene and SpCas9 mRNA (29.4%) and in 2 of 9 embryos after microinjections of guide RNA against *CD209* gene and SpCas9 mRNA (22.2%). Figures 1 and 2 present chromatograms of a number of embryo samples after microinjections of genetic constructs for making an incision in the *BLG* and *CD209* genes. It can be noted that the main sign of the presence of genetic modification is the double sequencing.

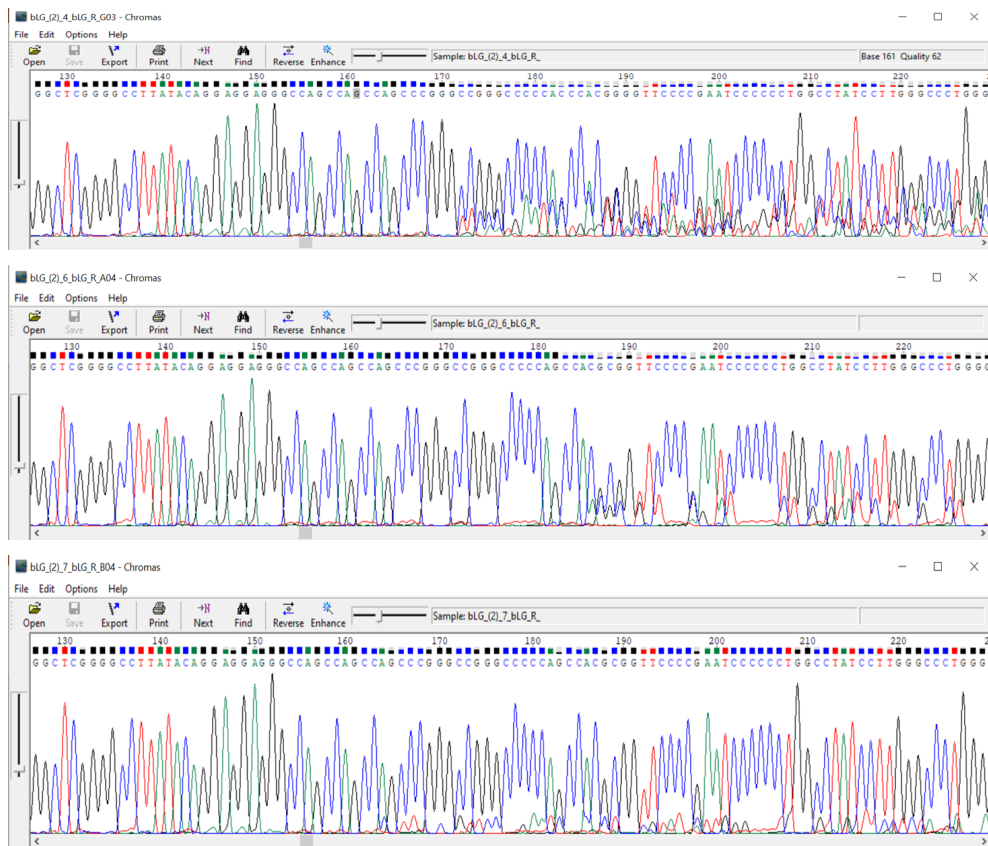


Fig. 1. Chromatograms received as a result of BLG gene fragment sequencing into which an incision was made to obtain a knockout.

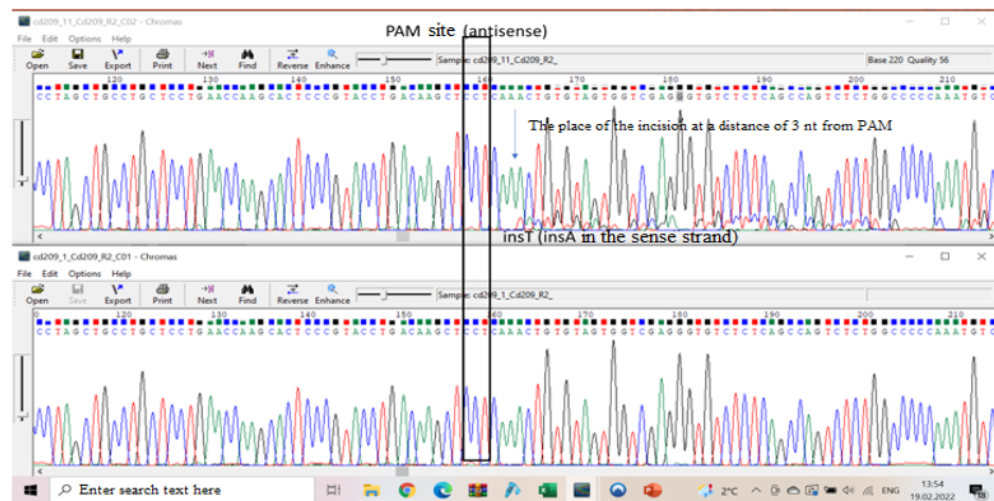


Fig. 2. Chromatograms received as a result of CD29 gene fragment sequencing into which an incision was made to obtain a knockout.

First of all, the data obtained testified to the success of the application of microinjections into the cytoplasm of the bovine zygote of the CRISPR/Cas9 editing system in the form of

RNA molecules. Secondly, we can see the efficiency of this delivery system, since 29.4% of the analyzed embryos after microinjections carried modifications of the *BLG* gene in the genome and 22.2% of the analyzed embryos after microinjections carried modifications of the *CD209* gene. Nevertheless, the resulting genetically modified embryos were mosaics. Presumably, this was due to the editing system running not at the zygote stage, but later. The high efficiency of genome editing promotes the planning of microinjected embryo transplantation experiments to produce animals with an edited genome.

4 Discussion

To improve the efficiency of gene editing in cattle, we have applied a delivery method of a genome editing system based on SpCas9 RNA and guide RNAs. Genome editing system delivery in the form of SpCas9 RNA and guide RNAs was conducted by microinjection of a solution of a genetic construct into a zygote. Following microinjections, the zygotes survived and began to split. Sanger sequencing of genes of interest in preimplantation bovine embryos and biopsies from them showed that 5 out of 17 embryos received after microinjections of guide RNA against the *BLG* gene and SpCas9 mRNA and 2 out of 9 embryos after microinjections of guide RNA against *CD209* gene and SpCas9 mRNA contain the necessary genome modifications. In this regard, the efficiency of the editing system for obtaining a knockout by the *BLG* gene was 29.4%. In the realm of the efficiency of the editing system for obtaining a knockout for the *CD209* gene was 22.2%.

The possibility of knockout of the b-lactoglobulin gene (the main allergenic milk protein) using ZFN was first proved by Yu et al [12]. Another group that reported deletion of the lactoglobulin gene in bovine embryos used TALEN [13]. Meanwhile, 1511 bovine zygotes were edited; the blastocyst growth rate was 15%, and the number of embryos carrying the deletion was 21%.

We achieved similar results using the CRISPR/Cas9 system: 20-30% of the knockout level and 16% of the blastulation level. Moreover, the frequency of blastocyst formation was comparable with the control (intact) group. Considering that bovine oocytes were obtained after slaughtering animals culled from farms due to age-related changes and low milk yield, and, accordingly, without prior hormonal training, the low level of competence for the development of such oocytes is quite understandable.

According to the sequencing results, all the genetically modified embryos obtained were mosaics, which, apparently, is due to the fact that genome editing does not originate at the zygote stage, when the editing system is delivered, but later. We did not perform an off-target analysis in the genome of the obtained embryos, since when editing the animal genome, the occurrence of non-targeted genome modifications is easily corrected in the process of obtaining a line of genetically modified animals when crossing with wild-type animals.

Therefore, the delivery of the editing system by microinjection of a mixture of SpCas9 RNA and guide RNAs is quite efficient and can be applied to produce knockouts by genes of interest. Nevertheless, further experiments with a large number of oocytes are also required to transfer the obtained embryos into the uterine cavity of recipient cows. The latter is intended to produce animals with an edited genome.

More generally, the obtained efficiency of knockout and embryo development currently suggests the potential use of CRISPR/Cas9 technology in the genetic modification of livestock.

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