Improvement of the technique for the bovine genome editing using the example of knockout of the CD 209 receptor responsible to the bovine leukemia virus

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Abstract. The aim of this work was to evaluate the possibility of editing the CD209 gene associated with bovine leukemia virus (BLV) infection. Two ways of delivering the gene editing system into the zygote were investigated: delivery of the CRISPR/Cas9 system using a viral vector and injection of mRNA and sgRNA into the oocyte cytoplasm. The most effective was the microinjection of spCas9 protein mRNA and sgRNA targeting CD209 at the zygote stage into the cytoplasm.

1 Introduction

One of the most widespread chronic infectious diseases of cattle in many countries of the world at present is enzootic leukosis. RNA-containing oncogenic bovine leukosis virus (BLV) serves as an etiological factor in the development of the infectious disease process. The causative agent belongs to the family Retroviridae, genus Deltaretrovirus, which also includes T-lymphotropic primate viruses (PTLV) - humans and monkeys (HTLV and STLV). This pathogen infects immunocompetent cells (T- and B-lymphocytes), initiates their malignant transformation and causes persistent lymphocytosis in 30-70% of infected animals, which develops in the terminal stage into severe multiple organ pathologies characterized by irreversible neoplastic processes.

Bovine leukosis virus is widespread and causes severe annual economic damage to the agricultural industry. Moreover, the virus may be associated with the development of breast cancer in women, as well as other neoplastic diseases of the hematopoietic system [1, 2, 3, 4]. Currently, the mechanism of pathogen transmission in such cases has not been studied, nor have the details of the selective tropicity of the virus been disclosed. A significant circumstance is that leukemia virus has close morphological and evolutionary similarities (58%) with human T-lymphotropic leukemia virus (HTLV). In general, BLV is extremely unlikely to be able to infect human cells. However, its similarity to HTLV does not exclude this probability [1, 5]. Recombination of BLV and HTLV may help to overcome species barriers, since it is known that replacement of the BLV RNA region containing the primary

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and secondary encapsulation signals with the HTLV homologous region makes it possible to obtain a recombinant virus capable of replicating in cell culture [6].

The main approaches used to control BLV are the detection and elimination or isolation of infected animals. Despite actively implemented anti-epizootic measures, the frequency of detection of infected hosts and deprived points remains constant, resulting in economic losses due to reduced production, export restrictions and the effects of immune dysfunction.

At present the only effective method of eradication of the disease in cattle population is implementation of complex programs of health improvement, including diagnostic tests of all cattle for antibodies to capsid proteins of gp 51 and p24 (ELISA, RID), or detection of virus genome fragments by PCR method, with following replacement of infected cattle. Positive results of this approach are disclosed and described in detail using the example of the Ural region (Sverdlovsk and Tyumen regions). The implementation of such programs requires a long time, on average 10-20 years and is associated with significant economic costs [7].

Mechanisms of bovine resistance to the leukosis virus are known. Genetic resistance is associated with the presence of allelic variants of the Bola - DRB3 gene encoding MHC class II in the genotype and is mediated by immune mechanisms. A polymorphism of the gene encoding the receptor required for BLV entry into body cells is also possible [8].

According to the literature data, the glycoprotein gp 51 (SU) is significant for the integration of the virus into the target cells. The secondary structure of the surface C-terminal part of BLV glycoprotein gp51 (SU) was shown to be significantly similar to that of the human Syncytin-1 protein. This glycoprotein is encoded by the ERVW-1 gene. The protein product of the gene (Syncytin-1) is expressed in the syncytiotrophoblasts of the placenta and is involved in the fusion of cytotrophoblast cells, resulting in formation of the syncytial layer. The ERVW-1 encoded glycoprotein Syncytin-1 has a similar structure to the envelope of retroviruses, including a furin cleavage site that separates the surface (SU) and transmembrane (TM) proteins, that form a heterodimer [10,11].

One of the receptors that Syncytin-1 (ERVW-1) interacts with is DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; CD209), which is a membrane protein, a product of the CD209 gene. This C lectin receptor is present on the surface of macrophages and dendritic cells [12]. The most important function of macrophage DC-SIGN is to recognize molecular patterns associated with pathogens, bind their proteins and then induce phagocytosis. In addition, myeloid and pre-plasmacytoid DC-SIGN is involved in interactions with endothelial cells and activates CD4+ T-lymphocytes. DC-SIGN is also known to have significant affinity for intercellular adhesion molecule 3 ICAM3 or CD 50. In addition, it has been found that DC-SIGN can modulate Toll-like receptors (TLR) and plays a role in dendritic cell-tumor recognition. The mechanism of DC-SIGN binding to various microorganisms is realized through recognition of glycoproteins containing mannose residues on their surface. DC-SIGN is also known to act as a receptor for certain viruses such as HIV (human immunodeficiency virus), HCV (hepatitis C virus). The interaction of these pathogens with dendritic DC-SIGN promotes the infection of T-lymphocytes [13,14]. The association of CD209 promoter polymorphism with HTLV (human T-cell leukemia virus) infection has been demonstrated previously [1,5,13]. There is an assumption that the DC-SIGN protein (CD209) can also act as a receptor for other retroviruses, not only BLV (bovine leukemia virus). In this regard, it is reasonable to investigate the possibility of modifying the receptor proteins using gene editing techniques, since successful modification can potentially provide the organism with resistance to viral infections whose pathogens are tropic to this receptor.

The aim of this work was to evaluate the possibility of editing the CD209 gene encoding the receptor associated with bovine leukosis virus infection.

2 Materials and methods

Two series of experiments on cattle genome editing were performed. Two methods of delivering the editing system into the zygote were investigated: delivery of the CRISPR/Cas9 system using a viral vector and injection of mRNA into the oocyte cytoplasm.

Donor biomaterial of cows was taken postmortem. Follicles were aspirated from the ovaries of cows under laboratory conditions, and oocytes were allowed to mature for 24 hours in BO-IVM culture medium (IVF-Bioscience) coated with mineral oil for cell culture (Sage), at a temperature of 38.5 C, carbon dioxide level of 6.5%, and oxygen of 5.0%. Cryopreserved bovine sperm was used for in vitro fertilization of oocytes. Spermatozoa were injected into matured oocyte-cumulus complexes at a concentration of 1.0-2.0 $x10^6$ motile sperm per 1 ml. Eighteen hours after insemination, the complexes were completely cleared of cumulus cells and spermatozoa. The presence of polar bodies was evaluated in the obtained oocytes. Oocytes that extruded the first and second polar bodies after fertilization were used to introduce the editing system.

Guide RNAs for the CD209 gene knockout were selected for the second exon of this gene using the online CRISPOR algorithm.

Adeno-associated viruses carrying a gene of green fluorescent protein, GFP (Green Fluorescent Protein) were used to study the delivery of the editing system by viral vector method. The optimal virus serotype was selected to infect cattle oocytes at the zygote stage: AAV1, AAV2, AAV6, AAV9, AAVDJ. The most optimal serotypes were used to deliver the editing system into the bovine zygote. In order to make site-specific breaks in the coding region of the CD209 receptor gene, a mixture of two components was used: a virus carrying sgRNA and a virus carrying spCas9. Adeno-associated virus at a concentration of 5×10^9 genomes of the virus in 100 µl of culture medium was added to Continuous Single Culture Complete culture medium (Irvine Scientific, USA). The culture medium was coated with sterile mineral oil (Oil for Tissue Culture (Sage, USA)) to prevent medium evaporation and pH changes. Bovine embryos at the zygote stage were placed in medium with adenovirus before 8-cell embryo stage at 38.5°C and 6.5% carbon dioxide level. Subsequent cultivation to blastocyst stage took place in medium containing no virus. The success of transduction was determined by the green fluorescence of protein when excited by blue light.

In the second series of experiments, breaks in genomic DNA were introduced at the zygote stage by microinjection of spCas9 protein mRNA and guide RNA (sgRNA) targeting Cd209 into the zygote cytoplasm.

In order to generate sgRNA *in vitro*, a plasmid was used in which the sgRNA sequence was under the control of the T7 promoter. The sgRNA was cloned into the pBluescript SK vector (Addgene vector database id 195), into which sgRNA-coding sequence for spCas9 was previously cloned at the XbaI and AscI sites with the possibility of cloning the recognition sequence of guide RNA at the two BbsI sites. BbsI-HF restriction endonucleases (New England Biolabs) was used for cloning; vector dephosphorylation was performed using FastAP enzyme (Thermofisher Scientific); purification in agarose gel and subsequent isolation was performed using Monarch DNA Gel Extraction Kit. During oligonucleotide synthesis, nucleotides were added to clone guide RNA targeting CD209 to be complementary to the cohesive ends formed when the vector was cut by BbsI-HF restriction endonucleases:

Cd209-T7-f TAGGCTCGACCACTACACAGTTTG

Cd209-T7-r AAACCAAACTGTGTAGTGGTCGAG

Oligonucleotides were phosphorylated using PNK kinase enzyme (Thermofisher Scientific), annealed with each other, and ligated with the previously prepared vector and

T4 DNA ligase enzyme (Thermofisher Scientific). The ligase mixture transformed into competent cells of XL1blue strain (Eurogen). Colonies were screened using PCR kits (Isogen), pSK T7 rev primers (5'-ttgtgtgatgctcgtcagg-3') and forward primer used for cloning. Two colonies each containing sgRNA were isolated using the Monarch Plasmid Miniprep Kit. The obtained plasmids were analyzed by Sanger sequencing. Sequencing of the obtained plasmids was performed using the primer pSK T7 rev.

The plasmid site containing the T7 promoter and sgRNA sequence was amplified with primers M13-forv (5'-gtaaaacgacggccagt-3') and gRNA-core-rev (5'-gcaaaagcaccgactc-3'). A PCR product of approximately 150 base pairs was reprecipitated and dissolved in water. RNA synthesis was performed using the MEGAshortscript™ T7 Kit (Thermo Fisher Scientific). The concentration of purified and isolated RNA was measured spectrophotometrically. Cas9 restrictase mRNA was obtained using the HiScribe™ T7 ARCA mRNA Kit (New England Biolabs). The site containing the open reading frame Cas9 under the control of the T7 promoter was amplified with the primers T7-f (5'-TAATACACGACTCACTATAGGG-3') and T7 term (5'-GCTAGTTATTGCTCAGCGG-3'). For synthesis 1 μg of purified PCR product was taken and incubated with kit components, then RNA was purified and isolated. The mix was prepared for injection into embryos with a cas9 mRNA concentration of 50 ng/μL and sgRNA CD209 of 25 ng/μL.

Injection of mRNA into the oocyte cytoplasm was performed on a Narishige micromanipulation unit (Japan). Zygotes were placed in G-MOPS Plus buffer (Vitrolife, Sweden) for the time of injection.

After injection of the editing system, embryos were transferred to BO-IVC culture medium (IVF-Bioscience) coated with mineral oil for cell culture (Sage). The embryos were cultured at a temperature of 38.5 C, carbon dioxide level of 6.5%, and oxygen level of 5.0% without changing the medium for the entire period of development up to the blastocyst stage.

Then the obtained embryos were evaluated. The following primer sequences were used to amplify the CD209 genomic fragment (fragment length 429 bp). CD209-F sequence 5'->3': TGAACACAAGGAGCCAGATGAC¸ CD209-R2 sequence 5'->3': GAAGAAGCCCAGTGAGACGA.

3 Results

In the first stage of the experiment with the viral vector delivery of an editing system, the optimal serotype of the adeno-associated virus was selected. The gene for the green fluorescent protein GFP was encoded into the viral genome. Transduction success was determined by the green fluorescence of this protein when excited by blue light (Figure 1). AAV1, AAV2, AAV6, AAV9, and AAVDJ serotypes were examined.

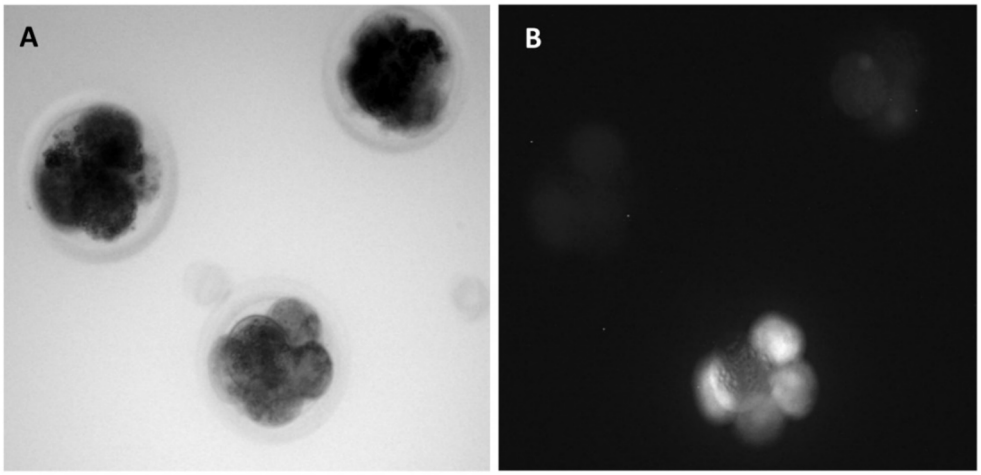

Fig. 1. Fluorescence of GFP protein in bovine embryos.

We estimated that the adeno-associated virus serotype AAV2 was optimal, so this serotype was used to infect cattle embryo cultures at the zygote stage as part of components carrying spCas9 and sgRNA targeting CD209 (Table 1).

Table 1. Results of optimal AAV serotype selection

Number of cells	AAV serotypes				
	AAV1	AAV2	AAV6	AAV9	AADJ
Beginning of cleavage	18	25	22	25	25
Blastocyst stage	3	8	3	3	8
Detection of green fluorescence	4	7	1	0	0

Thirty embryos were cultured after CD209 receptor editing; 14 embryos were obtained at the 8-cell stage, 3 of which reached the blastocyst stage (10%). The efficiency of editing the target genome site was on average 10.4%

About 200 cells were injected in total using the method of mRNA micro-injection into the cytoplasm of a fertilized oocyte. Cell death was about 10%. Almost half of the oocytes started cleavage, eight-cell stage reached 67.6%, and 11 embryos reached the blastocyst stage, which was 5.5% of all cells used (Figure 2).

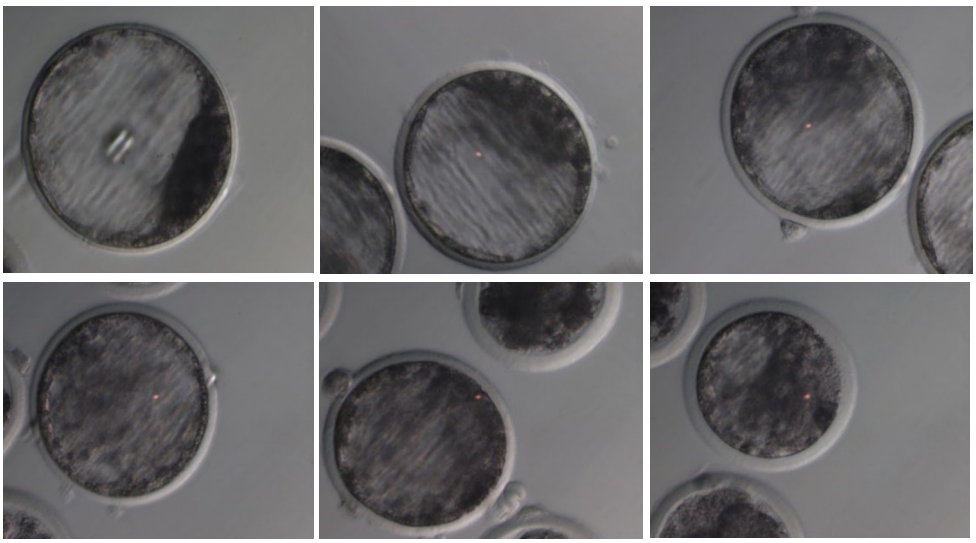

Fig. 2. Blastocysts obtained by model editing the CD209 receptor gene.

The development of the 32.3% zygotes stopped shortly after the start of cleavage. Cattle embryos that reached the preimplantation phase and their biopsy specimens were analyzed by Sanger sequencing of genes of interest. We found that after microinjection of guide RNA against the CD209 gene and spCas9 mRNA, the desired modification was present in the genome of 22.2% of the embryos (Fig.3). This indicates a high efficiency of this method for delivery of editing system delivery.

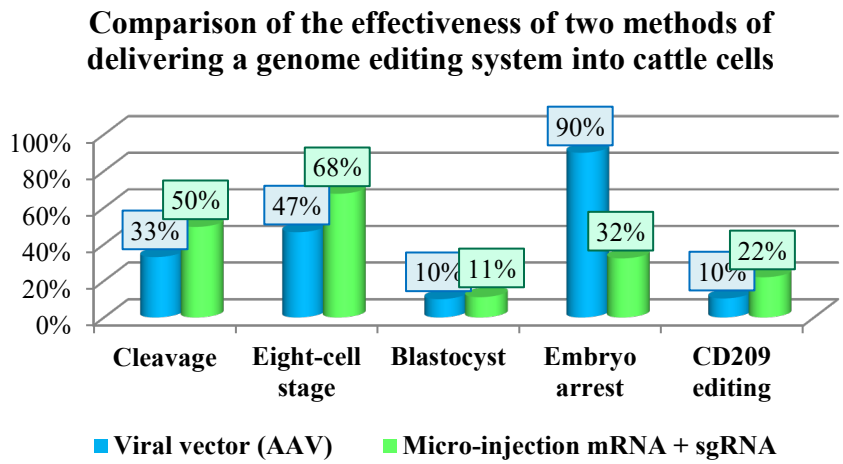

Fig. 3. Comparison of the effectiveness of two methods of delivering a genome editing system into cattle cells

4 Discussion

The studies showed that microinjection of mRNA and sgRNA was the most efficient way to deliver the CD209 gene editing system into a bovine zygote. When using AAV-based viral vectors, the AAV2 serotype showed the best results, but the overall efficiency of this

method was lower. It is worth noting that the proportion of embryos that matured to the blastocyst stage was relatively equal (10-11%).

Earlier, we tested another method of delivery. We performed microinjection of the gene editing system into the oocyte cytoplasm together with the sperm at the MII stage. For microinjection, we used plasmids based on the pX330 vector directing sgRNA to introduce breaks in the coding region of the CD209 gene. However, the work revealed that in cattle a single sperm microinjection was not sufficient to activate the oocyte, and often the zygote was not formed. In addition, DNA sequencing revealed that there were no changes in the embryo genome. The negative result was presumably due to the high lethality of microinjection of plasmid DNA into the cytoplasm. Thus, this method was technically very difficult due to the inability to visualize pronuclei. In addition, it also had zero efficiency in case of editing the Cd209 gene [15]. The method of viral vectors had relatively low efficiency and undesirable side effects. The most efficient was the microinjection of spCas9 mRNA protein and sgRNA targeting Cd209 at the zygote stage into the cytoplasm. However, all obtained embryos with the modified genome had a high degree of mosaicism, which is presumably due to the operation of the editing system not at the zygote stage, but later. Consequently, further improvement of the technique is required to reduce mosaicism. In addition, the receptor encoded by Cd209 has a number of functions unrelated to the interaction with the virus and associated with immunocompetent cell function. Therefore, knockout of the Cd209 gene can have side effects, up to and including immune system malfunction. In this regard, further comprehensive study of the mechanisms of this receptor is required for the rational and reasonable application of the genome editing tool for Cd209 modification.

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